CIRCUMVENTING OVICIDAL DEFICIENCIES OF FUMIGANTS DURING POSTHARVEST FUMIGATIONS

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CIRCUMVENTING OVICIDAL DEFICIENCIES OF FUMIGANTS DURING POSTHARVEST FUMIGATIONS

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Abstract: Sulfuryl fluoride (SF) is an increasingly used methyl bromide alternative for postharvest disinfestation of United States-produced walnuts. However, eggs of several key walnut pests are not adequately controlled by this fumigant at recommended label rate. These studies were conducted in the context of overcoming ovicidal deficiencies of SF during rapid field disinfestations. The first objective was to investigate the role of egg morphology in the context of species-specific fumigant efficacies. To accomplish this, the abundance, distribution, and location of respiratory openings on eggs of *Carpophilus hemipterus* (L.), *Tribolium castaneum* (Herbst), *Lasioderma serricorne* (F.), *Plodia interpunctella* (Hübner), *Ephestia elutella* (Hübner), and *Amyelois transitella* (Walker) were compared. Chorion structures and thicknesses of these species were also compared. Presence, distribution, and location of respiratory structures varied among species. This was the case for chorionic structures and thicknesses as well. The second objective was to establish dose responses of eggs of key walnut pests, namely, *C. hemipterus, T. castaneum, L. serricorne, P. interpunctella, E. elutella, A. transitella,* and *Cydia pomonella* (L.) to propylene oxide (PPO) in combination with 100 mmHg or carbon dioxide and normal atmospheric pressure. Results showed that PPO is an effective ovicide. Mortality tests on all insect species resulted in LC_{99} values ranging from 24.7-167.9 mg/liter at 100 mmHg and 4.0-17.3 mg/liter in combination with carbon dioxide. Corresponding CT (concentration x time) products were 49.3-674.4 mg h/liter and 95.3-414.8 mg h/liter, respectively. In general, coleopteran eggs were more tolerant to PPO compared to lepidopteran eggs, but *L. serricorne* was the exception. Findings from these studies suggest that species-specific ovicidal efficacies are possibly related to surface morphology of eggs, and that chorionic respiratory structure and chorion thicknesses may differentially affect fumigant penetration/uptake. PPO efficacy data provides information on concentrations of PPO required to kill eggs of key walnut pests at reduced and normal atmospheric pressure. These toxicity data represent a critical initial step in formulating a SF-PPO blend to meet postharvest disinfestation requirements of the California walnut industry. Future research should be aimed determining the absorbance and residue levels of propylene oxide and commercial testing of the blend.

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CHAPTER I

BACKGROUND AND OBJECTIVES

The annual production of dried fruits and nuts in the central valley of California is > 2,000,000 metric tons, with a value of ≈$18 billion. This accounts for nearly all of the dried fruits and nuts produced in the United States (USDA ERS 2013). California's walnut production in 2011 was valued at $1.34 billion, and $1.1 billion of this comprised exports (NASS 2013, USDA ERS 2013). However, several postharvest insect pests pose a serious threat to California's walnut production. Field pests that are of key concern in walnuts are *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) and *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Johnson et al. 2009, Burks and Johnson 2012). These two pests are quarantine pests in several countries that import in-shellwalnuts. Pests of concern during storage are *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae), and *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) (Johnson et al. 2009, Burks and Johnson 2012).

Postharvest management of insect pests is essential for the maintenance of a safe food supply and fumigation has a long history as a successful control measure. Chamber fumigation using methyl bromide (MeBr) is the most effective tool available to the California walnut industry to disinfest: incoming products during harvest, stored

products amenable to reinfestation, and outgoing product subject to required phytosanitary treatments. The walnut industry practices rapid throughput fumigation, i.e., MeBr for 24 h at NAP or for 4 h under low pressure for field disinfestations to accommodate large volumes of harvested walnuts in timely fashion. Low pressure fumigations are restricted to in-shellwalnuts destined for export. Given the declining use of methyl bromide (MeBr or CH_3Br) caused by regulatory phaseout, it is increasingly important to understand how various chemicals such as sulfuryl fluoride (SF or SF_2O_2), phosphine (hydrogen phosphide or PH_3), propylene oxide (PPO or C_3H_6O), ethyl formate (EF or $C_3H_6O_2$), ethanedinitrile (EDN or C_2N_2), controlled atmosphere (CA) gases, and non-chemical MeBr alternatives can be optimized to control key stored-product insect pests (Fields and White 2002, Throne et al. 2003, Johnson et al. 2012).

Sulfuryl fluoride (SF) is an increasingly used MeBr alternative in the United States for postharvest disinfestation. After the regulated phaseout of MeBr, the walnut industry transitioned to using SF. In scenarios where rapid disinfestations are required, i.e. high through-put fumigation for field disinfestations before marketing or storage, SF is used in combination with low pressure for 4 h or under normal atmospheric pressure (NAP) for 24 h. However, when used in these capacities, a drawback to using SF at the recommended label rate, i.e., 200 mg h/liter at low pressure or 1500 mg h/liter at NAP is that it is not effective against eggs of several pests of walnuts (S.W., unpublished data, UNEP 2011). Therefore ways to circumvent ovicidal deficiencies of SF in postharvest fumigations need to be found. A toxicological-based approach would be to blend SF with another fumigant that is highly effective against eggs.

Propylene oxide is a logical choice for blending with SF because it is a FDA approved sterilant for pasteurizing almonds. In fact, a 1:1 combination of PPO and SF has been shown to be effective against all life stages of *T. castaneum* (Muhareb et al. 2009). PPO has a very low environmental risk as an ozone depleter and is degraded into nontoxic propylene glycol in soil and in the human stomach (Griffith 1999, Navarro et al. 2004). However, its major disadvantage is flammability, which can be reduced by combining it with low pressure or CO_2 (8:92 – PPO:CO2) (Navarro et al. 2004). PPO at low pressure or in combination with CO_2 is toxic to eggs of *T. castaneum* and *P. interpunctella* (Isikber et al. 2004a, 2004b; Navarro et al. 2004; Isikber et al. 2006). Interestingly, these studies have shown that concentrations of PPO required to kill postembryonic stages of stored-product insects are much higher than those required to kill eggs, which is the opposite of SF that has ovicidal deficiencies but is highly effective against postembryonic stages.

Whereas developing a PPO:SF blend for effective control of all stages of stored-product insect pests is a good short-term solution to dealing with the ovicidal deficiencies of SF, it is important to have a long-term goal of elucidating why eggs respond differently to the same fumigant and to hopefully use this knowledge to improve fumigant efficacy. It is generally accepted that eggs of stored-product insect pests are the most fumigant-tolerant life stage and require higher concentration x time exposures for control compared with other stages (Bell 1976, Su and Scheffrahn 1990, Bell and Savvidou 1999, Baltaci et al. 2009, Bonjour et al. 2011, UNEP 2011, Athanassiou et al. 2012). Unlike other developmental stages (i.e. larvae, pupae, and adults) where the uptake of physiologically-active gases (e.g., oxygen, fumigant) occurs through the

tracheal system, which opens to the ambient environment via spiracles, gases enter insect eggs through respiratory openings such as aeropyles and micropyles found on the chorion, the outermost proteinaceous covering of the egg (Hinton 1963, Hinton 1981, Nation 2008). Aeropyles are microscopic holes in the chorion which extend radially inward, creating a network that allows, at least in theory, gas exchange with the ambient environment via the interstices of the inner chorion region (Tuft 1950, Hinton 1963, Daniel and Smith 1994, Trougakos and Margaritis 2002). A micropyle is a surficial invagination, through which sperm and gases enter the egg, located in various quantities at the anterior end, often in morphologically differentiated chorion termed the micropylar area (Tuft 1950, Outram 1967a, Arbogast et al. 1980, Arbogast and Byrd 1981, Margaritis 1985, Marvaldi 1999). Gases can also diffuse directly into the egg through the chorion (Tuft 1950, Outram 1967a, Trougakos and Margaritis 2002).

Eggs of different insect species respond differently to fumigants. In general, coleopteran eggs are more tolerant to fumigants compared to eggs of lepidopteran species (Mostafa et al. 1972, UNEP 2011, Walse et al. 2009). Variations in respiratory structures, chorion composition and thickness among different insect eggs may exist. The external morphology of insect eggs has been studied using the scanning electron microscope (SEM) in the context of differentiating species and tracing origins of infestations in domestic and international trade (Arbogast et al. 1980, Arbogast and Byrd 1981, Kučerová and Stejskal 2002, 2010, Hasbenli et al. 2008, Dutra et al. 2011, Baker et al. 2012). However, little or no work has been conducted to investigate the possible mechanistic relationship between respiratory openings on the chorion surface that facilitate gas exchange and the various environmental, physiological, and toxicological

factors that influence the relative tolerance of insect eggs to fumigants. In relation to SF, an increasingly used postharvest MeBr alternative in the United States, eggs of *C. hemipterus* (L.) require ≈30-, 33-, 12-, and 6-fold higher concentration of SF for control at the LD_{50} compared to eggs of *E. elutella*, *A. transitella*, *P. interpunctella*, and *T. castaneum* at ≈15.6°C (Baltaci et al. 2009, Walse et al. 2009, UNEP 2011). A 4-fold higher concentration of SF is require for the effective control of *C. hemipterus* eggs than for *L. serricorne* eggs at ≈26.7°C (Su and Scheffrahn 1990).

Objectives

The walnut industry has transitioned to using sulfuryl fluoride for disinfestations of postharvest pests. However, SF has ovicidal deficiencies and is ineffective against eggs several key pests when applied in high-throughput scenarios of 24-h fumigations at normal atmospheric pressure. This ovicidal deficiency of SF is even more pronounced in 4-h low pressure fumigations with the label rate restriction of 200 mg h/liter. As a result, high-throughput and quarantine and pre-shipment fumigations are still achieved by using methyl bromide allowed under critical use exemption (CUE) and quarantine and pre-shipment (QPS) exemption. However, CUE's for the walnut industry are not allowed after 2014. Because SF is the most likely postharvest alternative to MeBr, this work was conducted in the context of overcoming ovicidal deficiencies of SF during postharvest fumigations for continued use of this fumigant for postharvest disinfestations. The first approach taken was to explore how respiratory structures on the surface of the chorion may affect relative fumigant efficacy as an initial step in developing techniques to mitigate ovicidal deficiencies of fumigants in general, in the long term. The second approach was to evaluate toxicity of fumigant propylene oxide to determine

concentrations to blend with SF during postharvest fumigation to provide short-term solution to industry.

Therefore, the objectives of the current study are:

Objective 1.

To study egg morphology of key stored-product pests of the United States

Objective 2.

To study egg morphology and ultrastructure of chorions of key stored-product insect pests of the United States

Objective 3.

To determine the efficacy of propylene oxide against eggs of seven postharvest insect pests at low pressure

Objective 4.

To determine efficacy of propylene oxide in combination with carbon dioxide against eggs of six postharvest insect pests at normal atmospheric pressure

CHAPTER II

LITERATURE REVIEW

Dried Fruits and Tree Nuts

The fruits and tree nuts industries are an important component of the United States' farm sector averaging 13% of total crop revenue and generating more than $18 billion annually (USDA ERS 2013). The U.S. dried fruits and nuts industries are dominated by California growers and nearly all the dried fruits and nuts such as figs, prunes, raisins, walnuts, almonds, and pistachios are produced in the central valley of California. The United States leads the world in the production of tree nuts and the value of U.S. utilized nut production in 2012 was $7.4 billion (USDA ERS 2013). In terms of value, almonds are the leading horticultural commodity valued $4.35 billion, followed by walnuts ($1.34 billion), and pistachios ($879 million) (NASS 2013). Production values for the dried fruits dates, prunes, figs, and raisins for 2011 were ≈$43.9, 179.5, 20.3, and 863.7 million (USDA ERS 2013). Hong Kong is the largest importer of US-produced fruits and tree nuts (17.5%), followed by Spain (7.7%), Germany (7.1%), and India (5.7%) (USDA ERS 2013).

Walnuts

The United States is the world's largest exporter of walnuts. Annually, 470,000 tons of walnuts valued at $1.34 billion are produced in the central valley of California

(NASS 2013). More than 75% of these walnuts are exported and the export values of in-shellwalnuts and shelled walnuts in 2011 were $466 and $645 million, respectively. Hong Kong is the largest importer of walnuts followed by Germany, Turkey, South Korea, and Spain. Walnuts are relatively high-value products and are primarily used as snacks or as an ingredient in candies, cereals, and baked goods. Because these nutrition packed commodities are ideal for the development of insects and microbiological pests, postharvest infestation can seriously impact production, food safety, and subsequently profits.

Postharvest Insect Pests of Walnuts

Postharvest insect and microbiological pests can seriously affect walnut production, food safety, and subsequently profits. Postharvest insect pests of walnuts are broadly categorized into field and storage pests. Field pests are present at the time of harvest and are brought to storage where they continue to feed causing considerable damage and quality loss. *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) and *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) are the key field pests of walnuts. These two pests are of phytosanitary (quarantine pests) concern in several countries that import in-shellwalnuts. Other important field pests are *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae) and *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). Pests of concern during storage are *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae), and *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) (Johnson et al. 2009, Burks and Johnson 2012).

If not properly controlled, these insect pests can cause significant economic loss to the lucrative California walnut industry through direct damage by feeding, product contamination, and/or increased cost of control programs. A quarantine pest is defined as "a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled" (FAO UN 1996).

Table 1. Postharvest insect pests of walnuts

Common Name	Scientific name	Pest status
Field pests (do not normally reproduce in storage)		
Carob moth	*Ectomyelois ceratoniae*	Pest of dried fruits and nuts
Codling moth (CM)	*Cydia pomonella*	Quarantine pest in walnuts
Dried fruit beetle (DFB)	*Carpophilus hemipterus*	Field pest in dates, figs, and on drying fruits, Reproduces in storage under high moisture conditions
Navel orangeworm (NOW)	*Amyelois transitella*	Quarantine pest in California tree nuts and figs
Raisin moth	*Ephestia figulilella*	Found on drying fruits
Postharvest storage pests (primarily found in storage)		
Almond moth	*Cadra cautella*	Common in nut products
Indianmeal moth (IMM)	*Plodia interpunctella*	Most serious storage pest, common on all dried fruits and nuts
Tobacco moth	*Ephestia elutella*	
Grain and flour beetles	*Oryzaephilus* spp. and *Tribolium* spp. *Lasioderma serricorne*	Common in stored dried fruits and nuts

Source: Johnson et al. 2009, Burks et al. 2012.

The presence of quarantine pests greatly influences export trade with importing countries requiring quarantine treatments to prevent the introduction of exotic pests (USDA ARS 2010). Postharvest fumigation using methyl bromide is a key tool available for field disinfestations immediately after harvest, storage disinfestations to protect from storage pests, and quarantine and pre-shipment disinfestations to provide quarantine security. Quarantine security is based on the concept of Probit-9 that requires mortality of 99.9968%, so that upon transportation of the treated commodities, the targeted pests cannot become established in any area where they do not already exist (Aegerter and Folwell 2001). Methyl bromide (MB) is the most commonly used fumigant that consistently meets Probit-9 specifications. However, regulated phaseout of MeBr has diminished its availability for use.

Postharvest Pest Management in Walnuts

Field Disinfestation

As previously mentioned, pests of postharvest importance that infest in-shell walnuts in the field are *A. transitella* and *C. pomonella*. Walnuts are harvested in October/November and are immediately fumigated to control any incoming field pests before marketing or storage. Harvesting time of walnuts parallels with the highest demand time in domestic and international market, i.e. winter holiday markets. Therefore, any disinfestation practice adopted by producers and handlers should not preclude this market opportunity. For in-shell walnuts, importing countries mandate measures be taken to eliminate all life stages of *A. transitella* and *C. pomonella* to minimize the risk of introducing these exotic pests to their countries. Postharvest fumigation using MeBr is

the only effective measure that is practiced to disinfest walnuts of any field and storage pests that could be on them or are well hidden in the "meat" and are protected by the shell. Fumigations are conducted with 56 mg/liter MeBr for 4 h under reduced pressure or 56 mg/liter for 24 h at normal atmospheric pressure (Hartsell et al. 1991). However, methyl bromide has been phased out under the Montreal Protocol. After the phaseout of MeBr, sulfuryl fluoride (SF or SF_2O_2) was adapted as a replacement fumigant for low pressure fumigations to guarantee quarantine security under time sensitive scenarios. However, SF at its recommended concentration and exposure cannot control eggs of several pests that infest in-shell and shelled walnuts. It is important to note that because of the lack of a reliable alternative, the walnut industry still uses MeBr under critical use exemption (CUE) for disinfestation of in-shell walnuts under time sensitive scenarios and use of MeBr is also permitted under quarantine and pre-shipment (QPS) exemptions for providing quarantine security of walnuts that are exported to South Korea and Japan. However, CUEs for the walnut industry are not allowed after 2014 and QPS exemptions are political.

Storage Disinfestation

Stored walnuts are infested by various kinds of storage pests (Table 1). For shelled walnuts in storage, *A. transitella* and *C. pomonella* are not of major concern. These two pests are manually removed during the process of shelling. California's walnut industry does not use low pressure fumigation for stored walnuts because of the time flexibility associated with the marketing of shelled walnuts, i.e., no narrow time windows in which large quantities of product has to be fumigated in a short amount of time. Additionally, only ≈3 facilities in the central valley of California have the low pressure

fumigation infrastructure; and it is only practiced for disinfesting in-shellwalnuts destined for export. The industry practices fumigation using SF or phosphine at normal atmospheric pressure (NAP) for disinfesting shelled and/or in-shellwalnuts in storage.

Fumigants

Methyl Bromide

Methyl bromide has a long history as a successful fumigant in structural, commodity, and quarantine disinfestations for >50 yr. after its insecticidal properties were discovered by Le Goupil in 1930 (Fields and White 2002). MeBr was extensively used as the fumigant of choice for quarantine treatments especially for horticultural and durable commodities. During 1940s and 1950s, MeBr was considered the most effective fumigant in space treatments, especially in quarantine fumigations when control within 24 to 48 h was desired (Fields and White 2002). The extensive use of MeBr is a result of its ability to kill rapidly, not only insects and mites but also microflora and nematodes (Bell et al. 1996). It has high penetration ability, and can infiltrate commodities including wood; does not taint commodities; and is non-corrosive and inflammable (Bell et al. 1996, Bond 1994, White 1996). However, the discovery of MeBr as a serious ozone depleter has resulted in the phaseout of MeBr as fumigant (Aegerter and Folwell 2001, Fields and White 2002, Small 2007).

Phaseout of methyl bromide

The first event in the phasing out of MeBr was a result of work by Molina and Rowland (1974) that raised an alarm about the deleterious effects of chlorinated fluorocarbons (CFCs) on the stratospheric ozone layer. The ozone layer surrounds the earth from 19-23 km above the earth surface. It protects organisms by absorbing harmful

ultraviolet-B radiation, which if allowed can increase the risk of skin cancer and cataracts, reduce human and animal immune response, and hamper agricultural production. In 1985, evidence of the reduction of ozone was brought to international attention with the discovery of a "hole" in the ozone layer for part of the year over the Antarctic (Miller 1996). Bromine is believed to be an extremely efficient depleter of ozone, causing O_3 to lose the oxygen atom and become O_2 in repetitive actions (Butler and Rodriguez 1996). According to them, each bromine atom is capable of destroying 60 times more ozone molecules than a chlorine atom. In natural conditions where MeBr is produced, such as biomass burning and oceans, it is buffered by natural chemical reactions (Fields and White 2002) and artificial synthesis and use of MeBr was thought to have caused depletion of the stratospheric ozone layer. The extensive use of artificial MeBr for soil and commodity fumigation tipped the scales and together with the CFCs started a rapid decline in stratospheric ozone (Butler and Rodriguez 1996, Miller 1996).These findings propelled the "Montreal Protocol on Substances that Deplete the Ozone Layer", which was established in 1987 and today has been endorsed by most countries. The purpose of the Montreal Protocol is to eliminate substances that cause significant damage to the ozone layer. It took its first steps by reducing the emissions of chlorinated fluorocarbons and halogens, because these products release chlorine and bromine in the stratosphere, destroying the ozone layer. The follow up meeting in 1992 agreed MeBr was a significant ozone depleter and its use must be reduced (Fields and White 2002). The schedule for phaseout of MeBr in developed countries under the Montreal Protocol was: a) 25% reduction in 1999, b) 25% reduction in 2001, c) 20% reduction in 2003, and d) complete phase-out in 2005.

MeBr was initially to be phased out in the U.S. under the U.S. Clean Air Act of 1998 by January 1, 2001 (Aegerter and Folwell 2001). The 1999 Federal budget in the U.S. had made the U.S. phaseout identical to that required by the Montreal Protocol for developed countries allowing for exemptions that included QPS, CUEs, and emergency use exemptions (EUEs). QPS treatments are used to disinfest commodities from insect pests and to provide quarantine security. CUEs were established to allow the application of MeBr in situations where technically and economically feasible alternatives were not available. However, CUEs are not expected after 2015.

The existing infrastructure of the walnut industry that was developed over many years of successful MeBr use dictates fumigant application for postharvest protection. Given the declining use of MeBr caused by regulatory phaseout, it is increasingly important to understand how various chemicals such as sulfuryl fluoride, phosphine (hydrogen phosphide or PH_3), propylene oxide (PPO or C_3H_6O), ethyl formate (EF or $C_3H_6O_2$), ethanedinitrile (EDN or C_2N_2), controlled atmosphere (CA) gases, and non-chemical MeBr alternatives can be optimized to control key stored-product insect pests (Fields and White 2002, Throne et al. 2003, Johnson et al. 2012).

Alternatives to Methyl Bromide

Phaseout of MeBr warrants identification of effective alternative. Several factors are important in the search for an alternative to MeBr. The alternative(s) have to be efficacious and able to control all life stages of stored-product insects; they also have to be able to meet the environmental and regulatory requirements; and have the ability to elude the rapid development of resistance by pests (Aegerter and Folwell 2001). The alternatives must not alter the characteristics of the treated commodities or leave residues

that taint commodities. A very important factor is that the amount of time of treatment must be short. This treatment time must not significantly reduce the time available to market or completely preclude the opportunity to market the product in the most lucrative markets. The European markets do not require Probit-9 security, yet MeBr is still used to meet timeline restrictions, to provide U.S. quality standards, and to have the products in the European markets at a time of peak demand (Aegerter and Folwell 2001).

Several studies have been conducted to identify technically feasible MeBr alternatives (Aegerter and Folwell 2001, Fields and White 2002, Small 2006, Park et al. 2010). These include physical control methods, use of chemicals, and a combination of techniques – an integrated pest management approach (Aegerter and Folwell 2001, Fields and White 2002). Heat treatment is a commonly used physical management technique for controlling stored-product pests. Other physical management techniques include low temperature treatment, controlled atmosphere by the use of CO_2, irradiation, and low relative humidity (Fields and White 2002, Neven 2003). The key to developing physical quarantine treatments is to recognize the physiological weaknesses of the insect or to take advantage of the differences in the physiological responses of the commodity and the infesting insect (Neven 2003). This is because of the fact that insects usually do not exhibit the cross-protection phenomenon as plants do. For instance, many plants and fresh horticultural commodities respond to environmental stress in a stock manner, such that upon exposure to one stress, for e.g. a heat treatment, the plant becomes predisposed to withstand subsequent stress such as cold treatment (Neven 2003). Chemical alternatives that have been widely investigated include phosphine propylene oxide and sulfuryl fluoride (Aegerter and Folwell 2001, Isikber et al. 2004, Isikber et al. 2006,

Baltaci et al. 2008, Baltaci et al. 2009, Muhareb et al. 2009, Akan and Ferizli 2010, Hosoda 2010, Karakoyun and Emekci 2010, Park et al. 2010).

Sulfuryl Fluoride

Sulfuryl fluoride is registered as Profume® by Dow AgroSciences as a viable alternative to replace MeBr as a postharvest fumigant. It was first marketed as the structural fumigant Vikane™ (Dow AgroSciences LLC) in 1950, specifically for the control of drywood termites and wood-boring beetles (Small 2007). It was re-registered in the United States for post-harvest fumigation of cereal grains, dried fruits, and tree nuts in 2004. In fact, the walnut industry has transitioned to using SF during postharvest fumigations (Williams 2008). SF is a biologically active inorganic chemical that is odorless, colorless, non-corrosive, and non-flammable. It has a high vapor pressure (15.98 bar at 21.1°C) and low boiling point (-55.4°C) which allows for rapid diffusion and penetration (Tsai 2010). The mechanism of action is believed to be through the disruption of the glycolysis cycle leading to deficiency of metabolic energy (Cox 1997).

Several studies have demonstrated efficacy of SF towards postharvest insect pests; against postembryonic stages, SF is generally more toxic than MeBr for a given species (Kenega 1957). However, insect eggs are relatively more tolerant to SF than to MeBr, often requiring 4- to 54-fold higher concentration than that required to control adults of the same species (Reichmuth et al. 1997). The work by Outram (1967a, 1967b) suggests that the poor ovicidal activity of SF is mainly due to the impermeability of the protective eggshell structures. The proteinaceous eggshell and embryonic membranes chemically bind to SF limiting its activity (Outram 1967b). SF vacuum applications at the dose requirements listed on the current SF label (200 mg h/liter) cannot achieve complete

mortality of eggs of several walnut pests such as *A. transitella, C. pomonella, P. interpunctella, T. castaneum,* and *C. hemipterus* (S. W., unpublished data). In addition, the label rate of SF at NAP, 1,500 mg h/liter is not effective against eggs of several key walnut pests, namely, *C. hemipterus, T. castaneum, P. interpunctella* (UNEP 2011 and references therein).

Phosphine

Phosphine is a widely used fumigant throughout the world for controlling insects of durable commodities and is increasingly being studied as a potential alternative to MeBr (Field and White 2002). Phosphine holds promise as a replacement for MeBr fumigation against insect pests of dried fruits and tree nuts (Ferizli et al. 2007). In fact, several walnut processors use phosphine for disinfestation of stored walnuts. Phosphine is easily applied, inexpensive, and is amenable for use under many varying situations (Aegerter and Folwell 2001). However, it has several drawbacks that prevent it from being an ideal fumigant. Phosphine has slow activity (3-15 d), is flammable at concentrations above 1.8% by volume, it is carcinogenic, and causes corrosion of copper, silver, and gold (Fields and White 2002, Bond et al 1984). Most importantly, several stored-product pests develop rapid resistance towards phosphine (Nayak et al. 2003). Phosphine is a favored fumigant to replace MeBr for many uses, but the aforementioned practical difficulties must still be addressed.

Propylene Oxide

Propylene oxide (PPO) has been studied as another promising potential replacement for methyl bromide. PPO is a colorless liquid fumigant under normal environmental conditions with a boiling point of 34.2°C and ether like odor (Griffith

1999). This fumigant has very low environmental risk when compared to MeBr - it does not deplete the ozone layer and degrades into nontoxic propylene glycol in the soil and in human stomach (Isikber et al. 2006). It is a FDA-approved sterilant to kill bacteria, mould, and yeast contamination on processed spices, cocoa, and processed nutmeats except peanuts. The major disadvantage of PPO as a fumigant is its flammability, which ranges from 3-37% in air. This drawback can be overcome by PPO fumigation under low pressure or fumigating using a combination of PPO and carbon dioxide (CO_2). Several efficacy studies of PPO against stored-product insect eggs have shown that it is a potent ovicide (Isikber et al. 2004a, 2004b; Navarro et al. 2004, Isikber et al. 2006). For example, all life stages of *P. interpunctella* and *T. castaneum* were controlled with 13.9 and 19.9 mg/l, respectively, of PPO in empty chamber fumigation. Concentrations of 60.3, 72.1, and 93.1 mg/liter of PPO over a 4-h duration were required for 99% mortality *P. interpunctella* larvae when fumigated in presence of peanuts, almonds, and walnuts, respectively. Egg and larval stages of *P. interpunctella* were more tolerant and required higher concentrations- 15.3 and 13.9 mg/l PPO, respectively, to achieve LC_{99} values. Pupae and adults of *P. interpunctella* only required 8.8 and 5.9 mg/l PPO, respectively.

Absorption of PPO by commodities is relatively higher when compared to other fumigants. For example, walnuts absorb 91% of the initial concentration of PPO (Isikber et al. 2004), whereas methyl bromide absorption by wheat is less than 70% (Cherif et al. 1985). Amount of fumigant absorbed varies according to the type of commodity fumigated, moisture content, fumigant concentration, and dosage time (Isikber et al. 2006). What is most important is the residue that remains. Rapid desorption of PPO at normal atmospheric pressure 1 d after the termination of fumigation was observed which

implies PPO is physically bound to commodities and does not interact chemically (Isikber et al. 2004, Isikber et al. 2006). PPO residue level measured 1 d after termination of fumigation was 80 ppm for walnuts and this concentration is below the 300 ppm threshold, the maximum residue tolerance set by the FDA of the U.S. Therefore, a combination of PPO and low pressure has the potential to replace MeBr fumigation for CUE and QPS treatments. However, because of its flammability it should be applied either under low pressure or in combination with CO_2 to avoid combustion (Isikber et al. 2006). These studies show that PPO is an effective ovicide.

SF-PPO Blend

In order to overcome the ovicidal deficiencies of SF, the current recommendations are using repeat applications of this fumigant in order to target newly hatched larvae or to extend the exposure period beyond egg incubation time (UNEP 2011). However, SF fumigations in the United States are restricted by both dose requirements listed on the label and maximum residue limits (MRLs) on treated commodities (Johnson et al. 2012). The dose requirement listed on the current SF label for NAP and vacuum fumigations are 1,500 and 200 mg h/liter and the MRLs for inorganic fluoride and sulfuryl fluoride in walnuts are 10 and 3 ppm, respectively. Given the rapid disinfestation requirements associated with field disinfestations to accommodate large volumes of walnuts being harvested at the same time and timeline restrictions with the marketing of in-shellwalnuts and constraints imposed by the SF label dose and MRL thresholds, it is urgent that ways to overcome the ovicidal deficiencies of SF that take into consideration these three factors, be sought to facilitate its continued use for rapid postharvest disinfestations.

A toxicological-based approach to circumvent ovicidal deficiency of SF during postharvest fumigation is to blend SF with a potent ovicide (Muhareb et al. 2009). A 1:1 blend of SF:PPO has been shown to be effective against all life stages of *T. castaneum*. SF is the most likely MeBr alternative especially for rapid disinfestation purposes and is the only fumigant registered in the U.S. for low pressure fumigation (Walse et al. 2009). As previously mentioned, low pressure fumigations are critical for the export of in-shellwalnuts. High throughput fumigation is also achieved by fumigating with SF at NAP for 24 h. However, SF has ovicidal deficiencies when applied at the label rate, i.e., 1500 mg h/liter at NAP. This is even more pronounced at low pressure fumigations where the label rate is 200 mg h/liter at 100 mmHg. Therefore, blending SF with PPO during postharvest fumigation at low pressure or NAP seems like a logical choice.

Insect Eggs and Fumigant Efficacy

Several studies have shown that eggs are the most fumigant-tolerant life stage of stored-product insect pests and require higher doses for control than other life stages (Bell 1976, Hole et al. 1976, Su and Scheffrahn 1990, Walse et al. 2009, Baltaci et al. 2009, Athanassiou et al. 2012). For instance, and Su and Scheffrahn (1990) demonstrated that LC_{99} of SF required for control of eggs of cigarette beetle, *Lasioderma serricorne* (Fabricius) (Coleoptera: Anobiidae) was 32.35mg/l and that for larvae and adults were 2.54 and 1.59 mg/l, respectively. The relative tolerance of eggs has been attributed to reduced metabolic activity during embryo development (Bell 1976). This would imply a reduced amount of gaseous exchange or low fumigant penetration. Unlike other developmental stages, namely, larvae, pupae, and adults, where gaseous diffusion takes place through the tracheal system, which opens to the outside via spiracles, gaseous

diffusion in insect eggs is facilitated by respiratory structures such as aeropyles and micropyles (Nation 2008, Hinton 1981). Besides, there are variations in how eggs of different species respond to same fumigant (Mostafa et al. 1972, UNEP 2011). In general, coleopteran eggs are more tolerant to fumigants compared to eggs of lepidopteran species (Mostafa et al. 1972, UNEP 2011, Walse et al. 2009). For example, concentrations required to kill *C. hemipterus* eggs are 45 times greater than those required to kill *A. transitella* eggs, which can be effectively controlled in approximately one day at temperature above 15°C (Walse et al. 2009).

External morphology of insect eggs has been extensively studied using the scanning electron microscope (SEM) (Arbogast et al. 1980, Arbogast and Byrd 1981, Kučerová and Stejskal 2002, 2008, 2010, Hasbenli et al. 2008, Dutra et al. 2011, Baker et al. 2012). These studies have focused on elucidating external morphology of insect eggs in order to develop taxonomic keys for identifying species (Kučerová and Stejskal 2010). However, little or no work has been done to elucidate a possible mechanistic relationship between respiratory structures that facilitate gaseous exchange and relative tolerance of insect eggs to fumigants.

Insect Eggs

In most insects, except some viviparous aphids and flies, life begins as an independent egg. Insect eggs are centroletcithal, meaning that they have a central yolk, which provides nutrient to the developing embryo, surrounded by a layer of cytoplasm (Nation 2008). A vitelline membrane surrounds the cytoplasm and in most insects, a protective "shell" of protein secreted before oviposition by accessory glands in the female's reproductive system covers the egg. Each insect egg contains a living embryo

protected by an eggshell known as chorion, which is tough, rigid, and relatively water proof. In some insects, for example, in eggs of *Rhodnius prolixus* Stål (Hemiptera: Reduviidae), a thin layer of wax, which renders it impermeable, covers the chorion (Nation 2008).

Each egg is produced within the female's genital system and is eventually released from her body through an ovipositor. This process of egg formation and deposition is referred as oviposition and the organisms that lay eggs are known as oviparous. Every insect species produces eggs that are genetically unique and often physically distinctive as well, for instance in shape and size. Regardless of size or shape, each egg is composed of only a single living embryo. This embryo develops inside the protective chorion layer using the nutrients in the yolk and emerges out of the egg by breaking through the shell. The developing embryo inside the egg must obtain sufficient oxygen from the surroundings to complete its development (Nation 2008). There is very little information available on respiratory systems of insect eggs and the mechanism of respiration by a living embryo. According to Hinton (1981), the mechanism of respiration is diffusion that takes place through the aeropyles that dot the surface of the chorion. In some insects, the outer layer of the chorion is open to the atmosphere and aeropyles in the middle layer facilitate diffusion of respiratory gases through the middle layer to the embryo. In other insects, the outer layer of the chorion is dotted with aeropyles (Hinton 1981) which diffuses oxygen to the living embryo. However, some insect eggs do not have aeropyles (Arbogast and Byrd 1982, Kučerová and Stejskal 2008, 2010). In insects without aeropyles, exchange of respiratory gases takes place through the micropyler region or direct penetration of the chorion (Tuft 1950, Outram 1967a). The general lack of

information on the respiratory systems of insect eggs limits our understanding of the mechanism of resistance exhibited by some insect eggs. According to Outram (1967a), resistance is mainly due to the impermeability of the eggshell to the fumigant. The proteinaceous chorion layer of resistant *S. gregaria* binds to SF allowing only small amounts of fumigant to penetrate.

Respiratory Systems of Insect Eggs

Living organisms require a large surface area for oxygen absorption from the ambient atmosphere. However, organisms face a dilemma because water is lost in the process of acquiring oxygen because the water molecule is smaller than the oxygen molecule. Organisms have not developed a mechanism that simultaneously allows for oxygen acquisition while preventing water loss (Hinton 1981). Insects however, have been successful in dealing with the contradictory demands of the dry environments. The tracheas that ramify the insect body keep ample supply of oxygen, whereas the reduced area of spiracles and a continuous layer of wax on the cuticle prevent excessive water loss (Hinton 1981). The mechanism of respiration in insects is by simple diffusion (Hinton 1969, 1981). In adult insects, respiratory gases enter the body through the spiracle opening that is connected to the tracheal system. However, unlike adult insects, insect eggs do not have tracheal system for respiration.

According to Hinton, the majority of aquatic and semi-aquatic insects lay eggs with no specialized respiratory structures incorporated into the eggshell. On the other hand, the majority of terrestrial insect eggs are equipped with specialized structures for respiration, including an extensive inner chorionic meshwork that can function as plastron when the egg is submerged in water. The chorionic meshwork that holds a layer

of gas is equipped with small holes known as aeropyles. Aeropyles extend through the eggshell and allow for the continuity of the chorionic layer of gas with the surrounding atmosphere.

In eggs that have aeropyles that opens into a gas-containing meshwork in the chorion, it is possible that most of the gaseous exchange takes place through the aeropyles (Hinton 1981). Some eggs not only depend on the oxygen entering through the aeropyles but also use the oxygen that diffuses through the chorion. Tuft (1950) showed that eggs of *Rhodnius* were able to take up oxygen even when the aeropyles were blocked. However, rate of the diffusion was very low when compared with oxygen uptake by aeropyles. This experiment showed that oxygen can diffuse through the chorion. However, lack of information on the permeability of the chorion limits our understanding of other mechanisms of gaseous exchange in insect eggs. It is very important to understand the mechanism of diffusion of respiratory gases, or the uptake of respiratory gases by a live embryo in order to understand the mechanism of fumigant penetration. Outram (1967a) demonstrated that fumigant penetration in *S. gregaria* takes place mainly through the micropyler region whereas penetration through the chorion was common in *T. molitor*. Most SF binds to the proteinaceous layer of the chorion in *S. gregaria*, which may explain the resistance exhibited by *S. gregaria* eggs towards SF. Uptake and retention of fumigant by resistant eggs per unit time is relatively much lower compared to susceptible eggs (Outram 1967b). However, the amount taken up varies with the stage of embryonic development (Outram 1967a, Daniel and Smith 1994, Renthlei 2010). Therefore, the chorion of insect eggs plays a vital function in protecting the

embryo from external agents during development, while allowing for exchange of respiratory gases and entry of the sperm for fertilization (Woods et al. 2005).

Chorion and Choriongenesis

The insect eggshell is made of a vitelline membrane, chorion, wax layer, and the associated follicular epithelium that produces the constituent layers. Chorion synthesis is under the control of the 20-hydroxyecdysone synthesized by the follicular cells that surround the maturing oocyte (Belles et al. 1993). Follicular cells secrete chorion components and are responsible for the final chorion structures that may be remarkably complex involving more than one hundred proteins (Reger and Kafatos 1985). The chorion is largely proteinaceous, for instance the protein in the silkmoth chorion is 96% by weight. (Kawaski et al. 1971, 1972).

A mature follicle that further develops into an egg includes the oocyte surrounded by the vitelline membrane and the chorion that is approximately 20 µm thick. However, the thickness may vary within and among species (Kawaski et al. 1971). The thickness of the chorion of the household storage pest *Hofmannophila pseudospretella* Spuler (Lepidoptera: Oecophoridae) is 4.23 µm whereas; the chorion of another storage pest *Tenebroides mauritanicus* (L.) (Coleoptera: Trogostidae) is only 0.19 µm (Arbogast and Byrd 1982, Arbogast et al. 1984). Chorion formation involves sequential events that lead to the creation of a specialized layer by the follicular epithelium. The insect chorion has a number of layers that are unique and conserved in their composition as well as their disposition through the diverse insect groups. For instance, the mature chorion of the silkmoth *Bombyx mori* (L.) (Lepidoptera: Bombycidae) exhibits a tripartite ultrastucture - a trabecular layer closest to the oocyte, the inner lamellar layer, and the outer osmophilic

layer (Papankolaou et al. 1986). However, the vitelline membrane, the inner chorion layer, the endochorion, and the exochorion are the well-defined layers in the fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Margaritis 1985). It is possible that the relative composition of proteins determines thickness of the chorion leading to differences in external morphology of insect eggs. For instance, eggs of lepidoptera are highly sculptured (Arbogast and Browser 1989) whereas those of coleoptera have a somewhat smooth surface with faint imprints of polygonal follicular cells (Marvaldi 1999, Kučerová and Stejskal 2002) or are granulated (Kučerová and Stejskal 2008, Kučerová and Stejskal 2010). Heavy sculpturing of the lepidopteran eggs or the faint imprints of the coleopteran eggs might be a result of the final step in choriongenesis that takes place after ovipostion. This final step involves hardening of the eggshell that occurs through the process of protein cross-linking (Irles et al. 2011).

Aeropyles

Aeropyles are microscopic holes that dot the surface of the egg and extend radially into the chorion down to the inner layer (that holds a thin layer of gas) creating a network that allows gaseous exchange with the ambient atmosphere (Hinton 1981). Morphogenesis of aeropyles occurs simultaneously with choriongenesis and the localization, size, and structure of aeropyles are genetically programmed (Papanikolau et al. 1986). However, differences may occur even within individual mutants (Sakaguchi et al. 1973). During aeropyle formation in *Bombyx mori*, a bundle of microvilli extends through the follicle cells to the floor of the trabecular layer. As choriongenesis proceeds, the microvilli become longer and a cylindrical channel forms around them, which is filled with loose flocculent material, described as filler. Towards the end of choriongenesis,

localized secretion establishes the external rim of the aeropyle, thus forming a tiny hole within the ridge of cell imprint (Papanikolau et al. 1986). The locations, numbers, arrangements, sizes of the opening of aeropyles vary greatly among species. For instance, the eggs of *Philonthus immundus* Gyllenhal (Coleoptera: Staphylinidae) have 200 to 300 aeropyles scattered over the surface. On the other hand, aeropyles of *P. cruetatus* are arranged in rows - 8 rows, 4 with 15-18 aeropyles and 4 with 18-22 aeropyles. The aeropyles of *P. varians* have dumbbell shaped openings whereas openings of aeropyles in other species are circular (Hinton 1981). Great variations in relation to distribution, size of the opening, and number of aeropyles have also been demonstrated in the dipteran fruit fly, *Anasterpha* spp (Diptera: Tephritidae) (Dutra et al. 2011). *Anasterpha corollini* Carrejo and Gonzalez has 30 aeropyles with openings of variable diameters scattered on the ventral side of the egg. Another species, *Anasterpha distincta* Greene has 45 aeropyles of variable diameter. Distinctively, the ones with the larger diameters are concentrated on the ventral side and those with the smaller diameter are concentrated on the dorsal side. In both species the protuberances and polygonal structure on the surface of the chorion are not well developed. In another species of fruit fly, *Anasterpha zenildae* Zucchi, the chorion reticulation is well developed with different numbers of protuberances bordered by ridges and about 40 aeropyles of variable diameter are located at the vertices of the polygons. Structures of the aeropyles are an important diagnostic character used to distinguish stored-product moth eggs. Distinct collars surround the aeropyler openings of *E. elutella*, but those of *E. cautella* are unguarded (Arbogast et al. 1980). The size of aeropyle openings in *Plodia interpunctella* eggs is 2.1 µm whereas the size in *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae) is 1.2 µm.

Some beetles such as *Sitona* spp. Germer (Coleoptera: Curculionidae), *Cylydrorhinus farinosus* (Coleoptera: Curculionidae), *T. mauritanicus*, and *Platyaspistes argentinensis* Kuschel (Coleoptera: Curculionidae) are devoid of aeropyles (Arbogast and Byrd 1982, Marvaldi 1999). Other species such as *Enoplopactus lizeri* and *Naupactus rugosus* Kuschel (Coleoptera: Curculionidae) have few scattered aeropyles, whereas species such as *N. leucoloma* and *N. sulphurifer* have numerous aeropyles (Marvaldi 1999). It is likely that the variability in aeropyles is related to the habitat eggs are laid. Hinton (1981) suggested that plastron respiration is a common mechanism in many terrestrial insects that are subjected to occasional immersion in water after rain (occasional flooding). In insects with numerous aeropyles, the plastron facilitates gaseous exchange under these conditions by forming a bubble of air at air-water interface. Species like *N. leucoloma* and *N. sulphurifer* survive under wet conditions, but are also resistant to prolonged dry conditions (Marvaldi 1999). Air trapped in the plastron meshwork may also aid in preventing desiccation by slowing down the evaporation rate from the chorion surface (Hinton 1981). Interestingly, *C. farinosus* that lays its eggs in arid soil has a very smooth chorion surface without aeropyles. *P. argentinensis* lays its eggs protected between two leaf surfaces where the relative humidity is high. The chorion of this species has no other distinct opening except at the poles, and this may be micropyles. It is possible that these polar openings serve a respiratory function (Marvaldi 1999).The abundance, distribution, and sizes of aeropyle openings may have ecological significance in adaptation of insects to their environment because aeropyles are believed to play an important role in gaseous exchange between the living embryo and its outer environment (Hinton 1981).

Function of Aeropyles

Exchange of respiratory gases occurs through extensive airspaces that are located in the chorion (Hinton 1981). Renthlei (2010) observed significant changes in the size of aeropyle openings during the course of egg development. The size of aeropyles increased with the age of the egg up to 9 days and this may be due to high oxygen requirement by the developing embryo; a decrease in aeropyle size occurred closer to hatching. Daniel and Smith (1994) demonstrated an increase in oxygen uptake in *Calosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae) as the egg matured. Eggs were not able to develop when the only opening in the chorion, egg pore, was blocked. This may mean in *C. maculatus* exchange of respiratory gases only takes place through the egg pore, or oxygen diffusing through direct penetration of the chorion is insufficient to support development. Interestingly, they found the oxygen uptake rate in the Yemen strain of *C. maculatus*, which can overcome seed toxins, was double that of the Brazilian strain that is susceptible. This increased metabolic rate was related to the broader diameter of the egg pore in the tolerant strain. This may mean that in insects that have developed tolerance to toxins, the rate of gaseous exchange is higher allowing for increased metabolic rates, thus the number and/or the size of aeropyle openings may be significantly larger. For instance, *Drosophila grimshawi* Fallèn (Diptera: Drosophilidae) which has an unusually thick layer of chorion has developed numerous but very narrow aeropyles (Margaritis 1985). Based on these studies, greater number and/or size of aeropyle openings may enhance metabolic rate by allowing higher rate of gas exchange. On the other hand, eggs that require higher concentrations of fumigants may have developed mechanisms such as a reduced number of aeropyles or thick chorion to prevent penetration of fumigants.

Micropyles

A micropyle is a surficial invagination, through which sperm and gases enter the egg, located in various quantities at the anterior end, often in morphologically differentiated chorion termed the micropylar area (Tuft 1950, Outram 1967, Arbogast et al. 1980, Arbogast and Byrd 1981, Margaritis 1985, Marvaldi 1999). Contribution of micropylar area to gas penetration in insect eggs has been demonstrated (Tuft 1950, Outram 1967). Studies have also shown that the number of micropyles varies in and among species. Some insects have been shown to have only one micropyle (Dutra et al. 2011) whereas others have more than one micropyle and the numbers may vary among eggs of individuals of the same species (Tuft 1950, Kučerová and Stejskal 2010, Baker et al. 2012). According to Tuft (1950), the number of micropyles is related to the age of the female. Tuft (1950) states that aeropyles are the main site for gaseous diffusion and only one tenth of the diffusion takes place when all aeropyles are blocked, thereby suggesting diffusion may also take place through micropyles or the surface of the chorion. However, no differences in gaseous diffusion existed between the eggs laid by older and younger females which had varying numbers of micropyles (Tuft 1950), perhaps confirming that aeropyles are the main route through which gases diffuse into insect eggs. In *Rhodnius* egg, a thin and rigid fertilization membrane is secreted by the zygote after fertilization and together with the primary wax layer it seals the inner openings of the micropyle (Hinton 1981b); this would limit the role of micropyles in gaseous exchange. Contrary to the assertion that most diffusion takes place through the aeropyles, eggs of *Schistocerca gregaria* (Forsk) (Orthoptera: Acrididae) have been documented to allow maximum diffusion of fumigants such as SF through the micropyler complex (Outram 1967). These

seemingly contradictory conclusions suggest more studies are required to determine whether micropyles play any role in gaseous exchange in insect eggs.

Respiratory Horn

A respiratory horn is a projection of the chorion that has aeropyles concentrated at its apex and/or sides and functions as a gaseous exchange apparatus (Murrilo and Jiron 1994). In some eggs, the aeropyles are grouped together and open at the apex or open both at the apex and the sides of one or two respiratory horns that are usually long (Hinton 1981). Respiratory horns are common in the insect families, Miridae, Nepidae, Muscidae, Deraecorinae, Ranatatrinae, and Bryocorniae. The number of respiratory horns may vary greatly among families. For instance, the egg of Ranatatrinae has two respiratory horns whereas the egg of Nepidae may have four to 26 respiratory horns arising from the anterior pole (Hinton 1961). The number of aeropyles in respiratory horns varies greatly. Development of respiratory horns by fruit flies and bugs may have great significance in their evolution (Murillo and Jiron 1994). *Anastrepha obliqua,* a dipteran fruit fly has a short respiratory horn assembled with aeropyles at the anterior pole of its egg and is the only part exposed to the outer atmosphere, whereas the rest of the egg lies within the fruit pulp (Murillo and Jiron 1994). *C. hemipterus* is a serious pest of ripening and drying fruits and prefers to lay eggs during or after harvest. They do not attack sound fruits, but prefer overripe, fermenting, and rotten fruit (Burks and Johnson 2012). This may mean that *C. hemipterus* have developed respiratory appendages such as respiratory horns to facilitate gaseous exchange with the ambient atmosphere when the egg is still inside the ripening fruit.

Plastron

A plastron is a gas layer of constant volume with a large water-air interface that is held by a hydrofuge meshwork that resists the entry of water under hydrostatic pressure (Hinton 1967). In aquatic insects, the plastron is one of the major respiratory systems and is also called a permanent physical gill (Hinton 1976, Nation 2008). The plastron is common not only to aquatic insects; terrestrial insects eggs that are adapted to aquatic habitats also have well developed plastrons (Hinton 1981). A plastron enables an egg to extract oxygen from the ambient water, and therefore, continue development when flooded by rain (Hinton 1967). For instance, the eggshell of Muscidae has both an outer and inner meshwork layer, each of which holds a continuous layer of the gas. The middle layer provides mechanical strength to the eggshell. Aeropyles through the middle layer affect the continuity of the outer and inner layers of gas. Both layers consist of struts that arise perpendicularly to the middle layer and are branched at their apices in a plane normal to their long axes that forms a fine and open hydrofuge network that provides a large water-air interface when the egg is immersed in water. The peculiarity of this eggshell respiratory system is that it is adopted for both atmospheric and underwater respiration. Diffusion of gas through the aeropyles of the middle layer and through the network of the inner layer is unrestricted in air. In water, the outer meshwork provides a large water-air interface for the extraction of dissolved oxygen.

Ecological Significance of Egg Respiratory System

As previously mentioned, aeropyles, respiratory horns equipped with aeropyles at the tip, or plastrons are the three major types of respiratory systems found in insect eggs (Hinton 1981). The intrachorionic meshwork of the egg that holds a layer of gas is

connected to the ambient atmosphere via aeropyles. It appears that the differences in respiratory systems present in insect eggs are a function of their habitats. For example, eggs of terrestrial insects have evolved to have intrachorinoic meshwork that holds layer of gas between the interstices of the chorion and is connected to the ambient atmosphere via aeropyles (Hinton 1981). This type of respiratory system in terrestrial insect eggs allows gas exchange while preventing water loss or influx of water. In stored-product insect eggs that are laid in relatively dry environments, preventing water loss may be as important as allowing sufficient gas exchange for the developing embryo.

Although there is lack of information on the ecological significance of various respiratory systems, critical insights could be extracted from literature describing egg morphology of various pest species. Egg morphology of several pest species have been described with emphasis on surface sculpturing (Arbogast et al. 1980, Arbogast and Byrd 1981, Kucerova and Stejskal 2002, 2008, 2010, Dutra et al. 2011). These studies have shown that abundance, location, and size of the aeropyle openings vary greatly among species that are found in similar environments. For example, aeropyles in eggs of Tephritid flies are found at the tip of the respiratory horns that may vary in length (Murillo and Jiron 1994). Variations in egg respiratory systems also exist in stored-product insect eggs that are found in similar environment. For example, eggs of many coleopteran stored-product species that have been studied do not have aeropyles or micropyles whereas eggs of lepidopteran stored-product species have many aeropyles and micropyles (Arbogast et al. 1980, Arbogast and Byrd 1981, Kucerova and Stejskal 2002, 2008, 2010). It is possible that coleopteran and lepidopteran eggs may have evolved to have different mechanisms for gas exchange. Unlike eggs that have aeropyles where gas

exchange probably takes place predominantly through aeropyles, chorionic diffusion may be a major route of gas exchange in eggs that have very few or no aeropyles (Kucerova and Stejskal 2002). In addition, eggs of some species have well developed intrachorionic meshwork between the outer chorion and serosal cuticle (Hinton 1981). This intrachorionic meshwork may hold a greater portion of gas required by the developing embryo and therefore have fewer requirements for direct gas exchange with the ambient environment. On the other hand, it is also possible that this discrepancy could be a result of differences in the levels of microscopic details (magnifications) used in these studies and coleopteran eggs may have aeropyles with smaller aeropylar openings. However, it is evident that eggs of lepidopteran pests have wider aeropylar openings hence are easily visible under lower magnifications (Arbogast et al. 1980).

Gas exchange at any point in time for eggs of any given species could be a function of many factors, including the physiochemical interaction between the gas and the chorion, developmental stage of the embryo, development rate, and environmental parameters such as temperature and relative humidity. Since fumigant diffusion in insect eggs also takes place through the respiratory route, it is plausible that species with different respiratory systems may respond differently to fumigants. In addition, it is also possible that pest species subjected to selection pressure by fumigants may develop mechanisms to reduce fumigant uptake/penetration.

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CHAPTER III

EGG MORHOPLOGY OF KEY STORED-PRODUCT PESTS OF U.S.A

FORUM ARTICLE

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Egg morphology of key stored-product insect pests of U.S.A.

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Abstract

Eggs of *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae), *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae), *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae), and *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) were imaged with scanning electron microscopy to explore how respiratory openings on the chorion surface may affect the relative efficacy of fumigation. Each *C. hemipterus* egg had two aeropyles and no micropyles, *A. transitella* as well as *L. serricorne* eggs had many aeropyles and several micropyles, and each *E. elutella* egg had many aeropyles and a single micropyle. These data suggest that gases, including fumigants, differentially diffuse into the eggs of these species, with penetration through aeropyles and micropyles likely occurring to a greater extent in *L. serricorne*, *E. elutella*, and *A. transitella* than in *C. hemipterus.* Although confirmatory measurements of fumigant diffusion into eggs are needed, these findings suggest that species-specific ovicidal efficacies are related, at least in part, to the surface morphology of eggs and that chorionic respiratory structures differentially affect fumigant penetration and/or uptake.

Keywords Egg respiratory system, aeropyle, fumigant efficacy, diffusion, tolerance

Postharvest management of insect pests is essential for the maintenance of a safe food supply and fumigation has a long history as a successful control measure against stored-product insects. The U.S. dried fruit and nut, grain, processed food, and tobacco industries critically rely on postharvest chamber fumigation to disinfest: incoming products during harvest, stored products amenable to reinfestation, and outgoing product subject to a required phytosanitary treatment. Given the declining use of methyl bromide (MeBr or CH_3Br) caused by regulatory phaseout, it is increasingly important to understand how various chemicals such as sulfuryl fluoride (SF or SF_2O_2), phosphine (hydrogen phosphide or PH_3), propylene oxide (PPO or C_3H_6O), ethyl formate (EF or $C_3H_6O_2$), ethanedinitrile (EDN or C_2N_2), controlled atmosphere (CA) gases, and non-chemical MeBr alternatives can be optimized to control key stored-product insect pests (Fields and White 2002, Throne et al. 2003, Johnson et al. 2012).

It is generally accepted that eggs of stored-product insect pests are the most fumigant-tolerant life stage and require higher concentration x time exposures for control compared with other stages (Bell 1976, Su and Scheffrahn 1990, Bell and Savvidou 1999, Baltaci et al. 2009, Bonjour et al. 2011, UNEP 2011, Athanassiou et al. 2012). Unlike other developmental stages (i.e. larvae, pupae, and adults) where the uptake of physiologically-active gases (e.g., oxygen, fumigant) occurs through the tracheal system, which opens to the ambient environment via spiracles, gases enter insect eggs through respiratory openings such as aeropyles and micropyles found on the chorion, the outermost proteinaceous covering of the egg (Hinton 1963, Hinton 1981, Nation 2008). Aeropyles are microscopic holes in the chorion which extend radially inward, creating a network that allows, at least in theory, gas exchange with the ambient environment via

the interstices of the inner chorion region (Tuft 1950, Hinton 1963, Daniel and Smith 1994, Trougakos and Margaritis 2002). A micropyle is a surficial invagination, through which sperm and gases enter the egg, located in various quantities at the anterior end, often in morphologically differentiated chorion termed the micropylar area (Tuft 1950, Outram 1967, Arbogast et al. 1980, Arbogast and Byrd 1981, Margaritis 1985, Marvaldi 1999). Gases can also diffuse directly into the egg through the chorion (Tuft 1950, Outram 1967, Trougakos and Margaritis 2002).

The external morphology of insect eggs has been studied using the scanning electron microscope (SEM) in the context of differentiating species and tracing origins of infestations in domestic and international trade (Arbogast et al. 1980, Arbogast and Byrd 1981, Kučerová and Stejskal 2002, 2010, Hasbenli et al. 2008, Dutra et al. 2011). However, little or no work has been conducted to investigate the possible mechanistic relationship between respiratory openings on the chorion surface that facilitate gas exchange and the various environmental, physiological, and toxicological factors that influence the relative tolerance of insect eggs to fumigants. In relation to effectively managing key stored-product insect pests using SF, an increasingly used postharvest MeBr alternative in the U.S., eggs of *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae) require ≈30-fold higher concentration of SF for control at the LD_{50} compared to eggs of *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae), and *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) at ≈15.6°C (Baltaci et al. 2009, Walse et al. 2009, UNEP 2011) and ≈4-fold higher concentration than *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) at ≈26.7°C (Su and Scheffrahn 1990). Therefore, the abundance, distribution, and location of respiratory openings in the chorions of these four species

were compared as the first step in quantifying consequences of egg morphology on the relative tolerance of insect eggs toward fumigants.

Materials and Methods

Insects. Eggs were obtained from adult females of *C. hemipterus*, *A. transitella*, *E. elutella*, and *L. serricorne* reared in the insectary at the United States Department of Agriculture-Agricultural Research Service (USDA-ARS), San Joaquin Valley Agricultural Sciences Center, Parlier, CA. Voucher specimens of *C. hemipterus, L .serricorne, E. elutella,* and *A. transitella* adults which laid eggs used in this study were preserved in 95% ethyl alcohol and deposited at K. C. Emerson Entomology Museum at Oklahoma State University under lot numbers138, 140, 142, and 143, respectively. Rearing conditions for these insects were 27 ± 0.10°C (mean ± SE), 60 ± 0.24% RH (mean ± SE), and a photoperiod of 16:8 (L:D) h. *Carpophilus hemipterus* were reared on ripened banana on top of soil substrate in 946-ml glass jars; *L. serricorne* on rice bran diet in 946-ml glass jars; and *A. transitella* and *E. elutella* on red flaky wheat bran diet in 3.8-liter glass jars. The *C. hemipterus* culture was originally obtained in 1978 from Italian Swiss Colony Winery in Fresno county; *L. serricorne* in 1967 from an unknown source; *A. transitella* in 1966 from the University of California, Berkeley; and *E. elutella* in 1969 from USDA Tobacco Investigations Laboratory, Richmond, VA (USDA 2012).

Egg Collection. *Carpophilus hemipterus*. Freshly laid eggs, no older than 24 h, were obtained by placing 75 to 100 adults in a 237-ml glass jar. Glass slides (25 x 75 x 1 mm) were prepared as substrate for egg laying. A thin smear of coddling moth bean agar diet was spread down the center (the 75-mm midline) of each slide into which two wax paper strips (25 x 75 mm) folded in half were placed on each side with the crease folds

towards the center, i.e. covering the whole slide surface. Two slides were then bound together using a rubber band (#16) in such a way that wax papers (four of them) were sandwiched between the glass slides and the edges of the slides were aligned. Four prepared slide units were placed in a 237-ml glass jar along with egg-laying females. The glass jar was covered with moist filter paper, a wire screen (U. S. #40 mesh), a final moist filter paper covering, and all were secured using a threaded metal ring. Jars were placed in a holding room at 27 ± 0.01°C and 60 ± 0.24% RH for 12-18 h. Eggs were processed as described below for analysis using scanning electron microscopy (S-3500N Hitachi, High Technologies America, Pleasanton, CA).

Lasioderma serricorne. One hundred to 200 adults were aspirated into a 237-ml glass jar. The glass jar was covered with a wire screen (U. S. #40 mesh) and secured using a metal ring. The jar containing adults was inverted onto a 9-cm glass Petri dish lined with a filter paper and spaced (≈2-3 mm) from the filter paper using a large paper clip. Rice bran diet was used around the edge of the Petri dish to stimulate oviposition. The set up was placed in a holding room maintained at 27 ± 0.01°C, 60 ± 0.24% RH, and a photoperiod of 16:8 (L:D) h for 2-3 d. After 3 d, 0- to 3-d-old eggs were collected and analyzed using a SEM.

Ephestia elutella. One hundred to 200 adults were aspirated into a 1.9-liter glass jar that was covered with wire screen (U. S. #40 mesh) (USDA 2007). The jar containing adults was inverted over an old culture jar to discard any eggs and left on its side for few minutes. The jar was then inverted on top of a large paper clip spacer, which sat on a Petri dish (90 x 20 mm) lined with a filter paper. The egg layers were placed in a holding room maintained at 27 ± 0.01°C, 60 ± 0.24% RH, and a photoperiod of 16:8 (L:D) h for

2-3 d. After 3 d, 0- to 3-d-old eggs were harvested from the filter paper and analyzed using a SEM.

Amyelois transitella. One hundred and fifty adults were aspirated and transferred to a 2.3-liter coffee can and the can opening was covered with wax paper that was secured to the container with the help of a rubber band (#18). The set up was then placed on its side on an incubator shelf with the wax paper facing night light (4-watt bulb) in a holding room that was maintained at 27 ± 0.01°C, 60 ± 0.24% RH, and 16:8 (L:D) h for 2-3 d. SEM analysis was conducted on 0- to 3-d-old eggs.

Scanning Electron Microscopy. Freshly collected eggs (100-150) were mounted on double-sided carbon tabs (Ted Pella, Inc., Redding, CA) on aluminum stubs using a soft brush. *C. hemipterus*, *E. Elutella*, and *L. serricorne* eggs were first attached to a piece of single-sided tape. Subsequently, the tape containing eggs was then attached to a double-sided sticky carbon tab on an aluminum stub with eggs exposed. For *A. transitella*, the wax paper containing eggs was carefully cut into small pieces and attached to a double-sided sticky carbon tab on the aluminum stub with eggs exposed. To prevent specimen charging and increase secondary electron signals, eggs of all species were sputter coated with gold (SPI Module Sputter Coater) (SPI Supplies, West Chester, PA) at 5mA for 40 s at 90° to the target, followed by 40 s at 45° to the target. The samples were then viewed under a SEM and digital images were taken at 5.00 kV. When possible, the numbers of aeropyles on exposed surfaces of individual eggs were counted. In addition, pictures of entire eggs, chorion sculpture, aeropyles, and micropyles were taken; approximately 30 to 40 pictures each of different eggs, aeropyles, and micropyles were taken for each species. Measurements made on digital images using ImageJ

software (National Institute of Health, Bethesda, MD) included length and diameter (at widest point) of the egg, and diameter and cross-sectional area of each aeropyle opening. Each measurement was considered a replicate. External surface area and surface area-to-volume ratio (SA:V) were calculated. Parameters such as number of aeropyles per unit area of an egg and total aeropylar surface area were estimated, and where applicable, error was reported based on the propagation of standard errors (Lehrter and Cebrian 2010).

To test the null hypothesis that the diameters of aeropyle openings, external surface areas, or surface-to-volume ratios of eggs were equivalent across insect species, an analysis of variance (ANOVA) was conducted for each using Statistical Analysis System software (SAS Institute 2010) and the PROC GLM model. If the null hypothesis was rejected, the ANOVA was followed by Tukey HSD comparison tests to determine if means were statistically different (alpha = 0.05).

Cryofracturing *C. hemipterus* Eggs. A Hitachi S-3500N (Hitachi High Technologies, America, Pleasanton, CA) SEM equipped with a CT-1500 C cryo-unit (Quorum Technologies, East Grinstead, UK) was used to study the interior details of the chorion of cryofractured *C. hemipterus* eggs. Cryofracturing is a method previously applied to image cell interiors with SEM (Bozzola and Russell 1992). The eggs were aligned on a sticky tape and placed on the cryo-specimen holder, a copper stub with a groove along its diameter filled with a mixture of colloidal graphite and Tissue-Tek. The eggs were then cryo-fixed in nitrogen slush at its freezing point (≤ -210°C) for 30 s, transferred to the cryo-unit in the frozen state, where they were subsequently fractured, sublimated (15 min at -90°C), and sputter coated with gold at 7 mA for 2 min. The

fractured eggs were transferred to the cryostage of the SEM where they were analyzed at 15 kV and -178°C.

Results

General Egg Morphology. *Carpophilus hemipterus.* Eggs are cylindrical in shape (Fig 1A), 891.7-1,164.3 µm long, and 206.2-305.9 µm in diameter at the widest point (Table 1). Both anterior (narrower) and posterior (broader) ends of each egg are bluntly pointed. The surface of the chorion is smooth under low magnification (x100) (Fig. 1A). However, at higher magnifications (≥ 450x), the chorion surface has bumpy texture (Fig. 1B-D), and this is more pronounced at the anterior end. Two aeropyles, each located at the tip of the anterior end (Fig. 1C and D; Table 1), are funnel shaped and lack collars (Fig. 1E). A collar is thickened and raised chorion forming the rim of an aeropyle opening (Arbogast et al. 1980); it can be well developed or less distinct. Collars can be useful morphological features to facilitate identification of eggs of various insect species (Arbogast et al. 1980). The chorion has three distinct layers; a thick outer layer, a spongy middle layer, and a thin innermost layer (Fig. 1F). No micropyles were found.

Lasioderma serricorne. Eggs are ovate in shape (Fig. 2A), 347.2-433.2 µm long, and 179.1-247.5 µm in diameter at the widest point (Table 1). The egg gradually narrows toward the anterior and posterior ends, which are bluntly rounded (Fig. 2A and B). The egg surface, less the micropylar area prominently located at the tip of the anterior end (Fig. 2B and C), is marked by numerous tubercle-like projections (inconspicuous protuberances) that look granulated at lower magnification (250-400x) (Fig. 2A and B), but are clearly observed at higher magnifications (≥ 1,500x) (Fig. 2C and D - indicated by a circle). In addition to these tubercle-like projections, the anterior end has pillar-like

projections that are arranged in a pentagonal pattern (Fig. 2B - indicated by a square), which at higher magnification (8,000x) (Fig. 2D) are observed as a fibrous bundle that rises to a height of 11.21 ± 0.46 µm (mean ± SE) from the surface of the chorion. All chorionic projections, the tubercle-like projections, as well as pillar-like projections, have an aeropyle or aeropyles at the tips (8,000x and higher) (Fig. 2D and E; Table 1). The aeropyles lacked collars and were distributed over the egg surface. The micropylar area is located at the tip of the anterior pole and has 7-10 micropyles (Fig. 2 C and F; Table 1).

Ephestia elutella. Eggs are nearly globate in shape (Fig. 3A), 415.5-535.6 µm long, and 344.7-421.3 µm in diameter at the widest point (Table 1). The chorion is marked by numerous tubercle-like winding ridges, joined at termini, which did not have an angular pattern (Fig. 3A-C). Aeropyles, one or several, were present at the termini (Fig. 3C-E) and were distinctly characterized by a broad collar (Fig. 3E). Termini were distributed over the entire egg surface, but those with aeropyles were localized near the ends (Fig. 3C and D). At a high magnification (20,000x), the aeropyle opens to an inner chorionic meshwork (Fig. 3E). Approximately 10% of the observed eggs had a nipple-like projection formed by chorionic folds at the tip of the anterior end, which masked the presence of the micropylar area and a single micropyle opening (Fig. 3A - indicated by a white arrow). The ridges of the micropylar area were appressed, and there were 14-24 primary cells in a micropylar rosette that opened at the center (Fig. 3F). The primary cells are distinct loop-like or petal-like structures that have distinct ridges or carinae, and radiate out from the central depression at the apical end and give a flower like appearance to the micropylar region of the egg (Baker et al. 2012).

Amyelois transitella. Eggs are ovate in shape (Fig. 4A), 619.4-695.4 µm long, and 428.8-552.4 µm in diameter at the widest point (Table 1). The chorion is marked with protuberances that rise to form a reticulate pattern of polygons (Fig. 4A and B). Aeropyles were found at the vertices of polygonal structures and were distributed across the egg surface, although localized at the ends (Fig. 4C-E). Aeropyles were characterized by distinct broad collars and open to an inner chorionic meshwork (15,000x) (Fig. 4D). The polygonal ridges were prominent all over egg surfaces except in the micropylar area at the anterior end where 1-5 micropyles were centered at a rosette formed by 17-23 primary cells (Fig. 4E and F).

External Surface Area and Volume. External surface areas and SA:V ratios of eggs varied across species (F =513.2; df = 3,116; $P < 0.0001$ and F =503.9; df = 3,116; $P < 0.0001$, respectively; Table 2). The average external surface area of a *C. hemipterus* egg was significantly larger relative to *A. transitella, E. elutella* and *L. serricorne* eggs (Table 2). Likewise, the average external surface area of a *A. transitella* egg was significantly larger relative to *E. elutella* and *L. serricorne* eggs and the external surface area of a *E. elutella* egg was significantly larger than a *L. serricorne* egg. Among the insect eggs studied, *L. serricorne* had the smallest eggs (Tables 1 and 2). However, *L. serricorne* had the largest SA:V ratio compared to *C. hemipterus*, *A. transitella*, and *E. elutella* (Table 2). Similarly, *C. hemipterus* SA:V ratio was significantly larger relative to *A. transitella* and *E. elutella*, and *A. transitella* had larger SA:V ratio than *E. elutella*. However, estimate of *L. serricorne* external surface area is likely lower than the actual external surface area because elaborate chorionic sculpturing on the eggs was not accounted in the analysis (Fig. 2A-D).

Respiratory Structures. *Aeropyles.* Diameters of the aeropyle openings varied across species (F =74.81; df = 3,116; $P < 0.0001$; Table 3). The diameter of *C. hemipterus* aeropyles did not differ from those of *E. elutella* and *A. transitella* but was larger than those of *L. serricorne* (Table 3). The numbers of aeropyles per μm^2 in *L. serricorne*, *E. elutella*, and *A. transitella*, estimated by dividing the average number of aeropyles per egg by the average external surface area of an egg, were ≈435,000-, 15-, and 5- fold greater, respectively, than in *C. hemipterus*.

Carpophilus hemipterus eggs had only 2 aeropyles each and each aeropyle opening was 1.24 ± 0.11 µm (mean ± SE) in diameter (Table 3). The number of aeropyles per μm^2 was $2.0 \times 10^{-6} \pm 3.5 \times 10^{-8}$ (mean ± SE) (Table 3). The combined area of all the aeropyle openings per egg, the total aeropylar surface area, was 2.16 ± 0.19 μm^2 (mean ± SE) based on estimation by multiplying the average cross-sectional area of an aeropyle and the average number of aeropyles per egg (Table 3). Relative to the other species, the area of chorion open to ambient atmosphere (total aeropylar area + micropylar area) was smallest in *C. hemipterus* and was ≈491.9-, 9.6-, and 2.54-fold less than that for *L. serricorne*, *E. elutella*, and *A. transitella* eggs.

Lasioderma serricorne had 291,200 ± 13,145 (mean ± SE) aeropyles per egg. These aeropyles were 0.10 ± 0.01 µm in diameter (Table 3), significantly smaller in diameter than *C. hemipterus*, *E. elutella*, and *A. transitella* aeropyles (Table 3). The estimated number of aeropyles per μm^2 of *L. serricorne* egg surface was 0.87 ± 0.043, which is ≈435,000-fold greater than that of *C. hemipterus* and at least 5 orders of magnitude higher than observed for the other species. The total aeropylar surface area,

990.1 ± 180.4 μm^2, was estimated by multiplying the average cross-sectional area of an aeropyle opening by the average number of aeropyles per egg.

Ephestia elutella had 17.4 ± 0.79 aeropyles per egg, all localized at the ends (Table 3). The number of aeropyles per μm^2 for *E. elutella* was ≈15-fold greater than for *C. hemipterus* (Table 3). The diameter of *E. elutella* aeropyle opening was 1.40 ± 0.07 µm and was significantly greater compared to other species (Table 3). In addition, the estimated total aeropylar surface area was 20.71 ± 1.39 μm^2 (Table 3).

Amyelois transitella had 7.24 ± 0.36 aeropyles per egg with their distribution predominantly at the ends with a few in the midsection (Table 3). The number of aeropyles per μm^2 for *A. transitella* was ≈5-fold greater than for *C. hemipterus* (Table 3). The diameter of a *A. transitella* aeropyle opening was 1.07 ± 0.04 µm and was not different from that of a *C. hemipterus* aeropyle opening but was larger than that of *L. serricorne* and smaller than that of *E. elutella* aeropyle opening (Table 3). The estimated total aeropylar surface area was 5.50 ± 0.34 μm^2 (Table 3).

Micropyles. The diameter of each micropylar opening of a *L. serricorne* egg was 3.04 ± 0.09 μm^2 (mean ± SE) and the estimated total micropylar cross-sectional area was 72.5 ± 4.2 μm^2 (mean ± SE), calculated by multiplying the average area of each micropyle by average number of micropyles per egg, which provides 14-fold less surface area for gas exchange relative to that provided by aeropyles (aeropylar area). The micropylar surface area to route gas exchange was negligible (<1%) in *A. transitella* and *E. elutella* eggs and *C. hemipterus* eggs had no micropyles.

Discussion

The present study provides, for the first time, quantitative information on morphological and physical factors that influence gas exchange in the eggs of key stored-product insect pests (*C. hemipterus*, *L. serricorne*, *E. elutella*, and *A. transitella*), such as the number of aeropyles present, aeropylar area, external surface area, the percentage of external surface area covered by chorion, and estimated egg volume. Descriptions of the external morphology of *E. elutella* and *L. serricorne* eggs are generally consistent with those previously described by Arbogast et al. (1980) and Kučerová and Stejskal (2010), respectively. However, this study notes that whereas Kučerová and Stejskal (2010) indicated there were no aeropyles present in *L. serricorne* eggs, my examination of these eggs showed that they have aeropyles. This discrepancy could be a result of differences in the strains of *L. serricorne* studied and the level of magnification used in these two studies. In the present study, aeropyles in *L. serricorne* were observed at a magnification of 8,000 and higher (Fig. 2D and E), whereas Kučerová and Stejskal (2010) used a magnification of 3,700. Descriptions of external morphology of eggs from the family Pyralidae were similar to that described above for *E. elutella* and *A. transitella*, having a sculptured chorion marked with tubercles that are joined together to form an angular pattern or winding ridges without any angular pattern, a micropylar area surrounded by cells that form a rosette like structure, and several aeropyles with distinct collars distributed all over the surface of the chorion but predominant at the ends (Arbogast et al. 1980, Arbogast and Byrd 1981, Baker et al. 2012). There are currently no published SEM descriptions of eggs from the family Nitidulidae, which includes *C. hemipterus*. Descriptions of external egg morphologies of various stored-product pests are useful

tools in identifying species and tracing origins of infestations in domestic and international trade (Kučerová and Stejskal 2010).

Morphological and physical characteristics of eggs were quantified to estimate the relative potential contribution of chorionic respiratory structures to gas penetration and/or uptake. Respiratory structure-normalized surface areas (aeropylar and micropylar areas) were calculated to estimate the amount of egg surface area available for passive uptake of gases, which precludes the diffusion of gas through the chorion. The average diameter of the opening of aeropyles for the four species studied ranged from 0.1-1.39 µm, at least three orders of magnitude greater than the size of O_2 (1.2 Å), MeBr (2.49 Å), SF (2.99 Å), PH_3 (2.05Å), ozone (O_3) (1.42 Å), and PPO (2.11 Å), and are expected to easily accommodate the mean free path of gas molecules (1000 Å) (Hinton 1963) under conventional fumigation conditions. The total aeropylar surface area to route passive gas exchange (Tuft 1950) was highest in *L. serricorne* eggs and lowest in *C. hemipterus*. For *L. serricorne*, the micropylar area is 7% of the aeropylar area, suggesting that passive gas diffusion via this route would be concomitantly less. For the other three species, micropyles are not likely an appreciable route for passive gas diffusion.

While diffusion of gases through the chorion of insect eggs has not been directly probed, critical insight into the mechanism of gas diffusion through lipidic and cellulosic films (surfaces) can be extracted from literature detailing the kinetics of gas diffusion into fruits (Walse et al. 2013). Diffusion through the chorion would be a function of many factors, including physiochemical interaction between the gas and the chorion, the thickness of the chorion, the surface area of the chorion, and the surface area to volume ratio of the egg. Presumably, in eggs with fewer or no respiratory openings, the

contribution of diffusion through the chorion to the overall uptake of gases would be greater compared to eggs with relatively larger aeropylar and micropylar areas.

Enumerating the relative contribution of chorionic diffusion versus respiratory structure-mediated uptake of gases is critical to understanding how egg morphology could be related to the relative ovicidal tolerance of insect species towards fumigants. Future research will be aimed at systematically and quantitatively exploring the role of egg morphology and molecular diffusivity, using microscopic and molecular marker techniques, in the context of species-specific fumigant efficacies.

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Table 1. Parameters (mean ± SE) and descriptions of eggs and location, abundance, and attributes of respiratory openings of *C. hemipterus*, *L. serricorne*, *E. elutella*, and *A. transitella* eggs (*n* = 30).

Attribute	*C. hemipterus*	*L. serricorne*	*E. elutella*	*A. transitella*
Length (µm) (Range)	1063.2 ± 12.5 (891.7-1164.3)	400.2 ± 3.7 (347.2-433.2)	488.1 ± 4.6 (415.5-535.6)	651.1 ± 4.6 (619.4-695.4)
Diameter (µm) (Range)	264.9 ± 3.6 (206.2-305.9)	210.5 ± 3.1 (179.1-247.5)	373.5 ± 3.6 (344.7-421.3)	476.9 ± 6.5 (428.8-552.4)
L/W ratio	4.03 ± 0.07	1.91 ± 0.03	1.31 ± 0.02	1.37 ± 0.02
Surface of the chorion	Smooth	Basal tubercle-like projections; pentagonal pattern of pillar-like projections at the anterior end	Tubercles joined together by ridges at the termini	Reticulate pattern of polygons
Distribution of aeropyles	Anterior end	Distributed over the surface of egg	Distributed at ends; predominant at the posterior end	Distributed over the surface of egg; predominant at ends
Micropyles	Absent	Present; 7-10 micropyles	Present	Present; 1-5 micropyles

Table 2. Surface area and volume parameters (mean ± SE) of *C. hemipterus*, *L. serricorne*, *E. elutella*, and *A. transitella* eggs (*n* = 30).

Insect Species	External surface area of an egg (μm^2)[a]	Surface-to-volume ratio[b]
C. hemipterus	$9.9 \times 10^5 \pm 1.8 \times 10^4$a	$17.0 \times 10^{-3} \pm 2.2 \times 10^{-4}$b
L. serricorne	$3.4 \times 10^5 \pm 6.5 \times 10^3$d	$24.1 \times 10^{-3} \pm 2.9 \times 10^{-4}$a
E. elutella	$5.8 \times 10^5 \pm 8.8 \times 10^3$c	$13.9 \times 10^{-3} \pm 1.1 \times 10^{-4}$d
A. transitella	$6.8 \times 10^5 \pm 1.2 \times 10^4$b	$14.9 \times 10^{-3} \pm 1.3 \times 10^{-4}$c

[a]Means within a column followed by different letters are significantly different ($\alpha = 0.05$).

[b]Means within a column followed by different letters are significantly different ($\alpha = 0.05$).

Table 3. Aeropylar parameters (mean ± SE) of *C. hemipterus*, *L. serricorne*, *E. elutella*, and *A. transitella* eggs (*n* = 30).

Insect Species	Diameter of aeropyles (Range) (µm)[a]	Cross-sectional area of an aeropyle (μm^2)[b]	Number of aeropyles per egg (*n* = 40)	Number of aeropyles per unit area (μm^2) of an egg	Total aeropylar surface area per egg (μm^2)
C. hemipterus	1.24 ± 0.11ab (0.49-2.58)	1.08 ± 0.15	2 ± 0.00	$2.0 \times 10^{-6} \pm 3.5 \times 10^{-8}$	2.16 ± 0.19
L. serricorne	0.10 ± 0.01c (0.034-0.204)	0.0034 ± 0.0006	291,200 ± 13,145	0.87 ± 0.043	990.1 ± 180.4
E. elutella	1.40 ± 0.07a (0.82-2.55)	1.19 ± 0.20	17.4 ± 0.79	$2.9 \times 10^{-5} \pm 1.4 \times 10^{-6}$	20.71 ± 1.39
A. transitella	1.07 ± 0.04b (0.71-1.66)	0.76 ± 0.05	7.24 ± 0.36	$1.06 \times 10^{-5} \pm 5.6 \times 10^{-7}$	5.50 ± 0.34

[a]Means within a column followed by different letters are significantly different.

[b]Cross sectional area of aeropyles was not calculated directly from average diameter or radius of the aeropyles but as described in the materials and methods section.

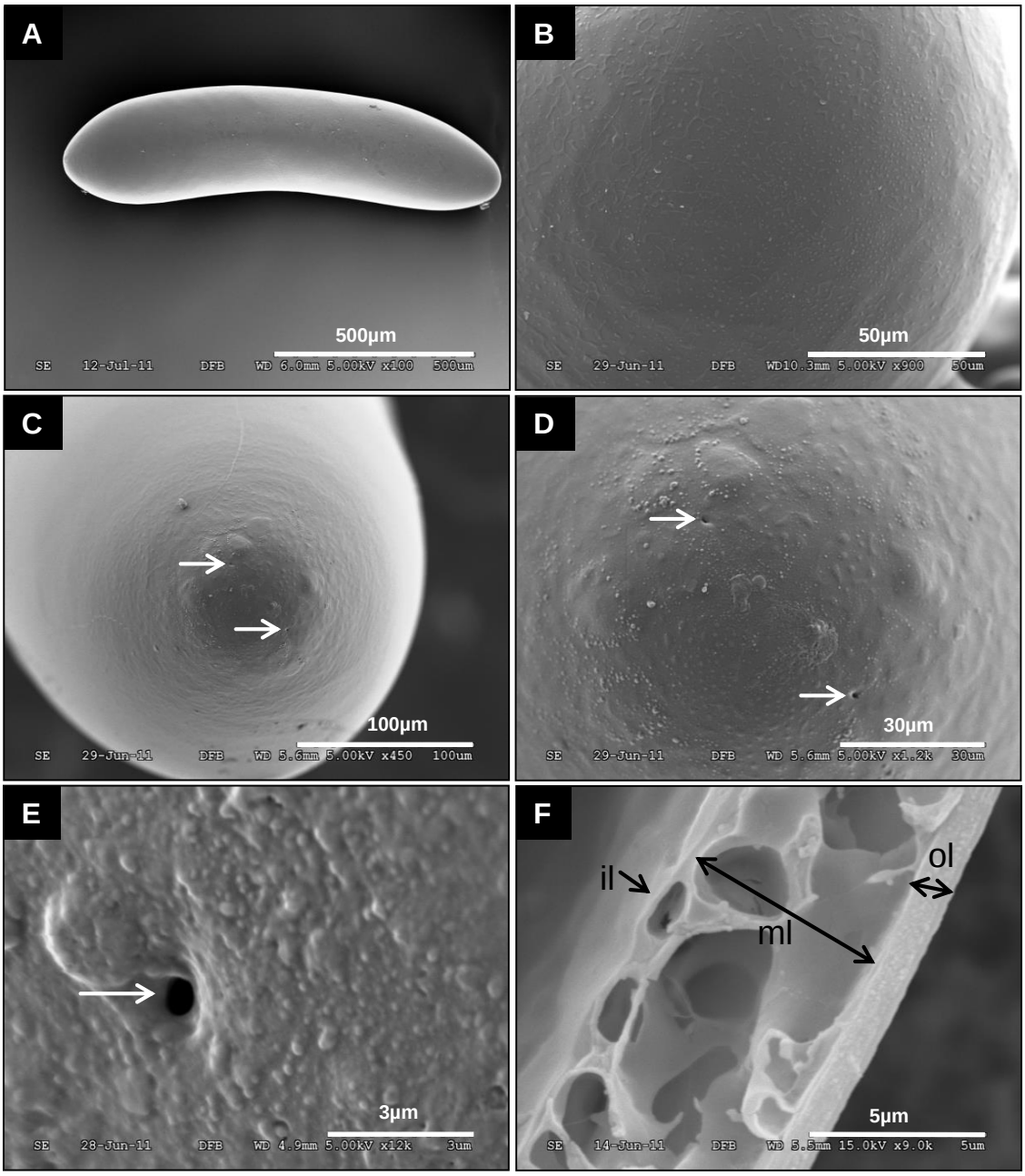
Figure Caption

Fig. 1. Scanning electron micrograph of a *C. hemipterus* egg (100x) (A), a bumpy texture of the egg chorion (900x) (B), anterior end of the egg with aeropyles indicated by arrows (x450) (C) and at 1,200x (D), a magnified aeropyle (12,000x) (E), and a cross-section of the freeze fractured chorion – ol is the outer layer, ml the middle layer, and il the inner layer (F) (9,000x).

Fig. 2. Scanning electron micrographs of a *L. serricorne* egg (250x) (A), the view of an egg from the anterior end showing chorionic sculpture (400x) (B), the anterior end with micropyles – note the 9 micropyles (1,500x) (C), an aeropyle at the tip of a chorionic projection indicated by a white arrow (8,000x) (D), aeropyles at the tip of a chorionic projection indicated by white arrows (40,000x) (E), and a magnified micropyle (15,000x) (F).

Fig. 3. Scanning electron micrographs of a *E. elutella* egg (200x) (A), sculpture of the posterior-end chorion (400x) (B), the posterior end of the egg with aeropyles indicated by black arrows (700x) (C), aeropyles atop a single chorionic projection (5,000x) (D), a magnified aeropyle showing the inner chorionic meshwork (20,000x) (E), and the anterior end of the egg with a micropyle indicated by a white arrow and aeropyles indicated by black arrows (1,500x) (F).

Fig. 4. Scanning electron micrographs of a *A. transitella* egg with a white arrow pointing at the micropylar location (150x) (A), the posterior end of an egg showing polygonal sculpture of the chorion (700x) (B), aeropyles at the vertices of polygonal structures indicated by black arrows (1,500x) (C), a magnified aeropyle showing the inner chorionic meshwork (15,000x) (D), the anterior end of the egg with the micropylar area indicated by a white arrow and aeropyles by black arrows (1,500x) (E), and the location of 5 micropyles indicated by a white arrow (4,000x) (F).


A
500µm
SE 12-Jul-11 DFB WD 6.0mm 5.00kV x100 500um
B
50µm
SE 29-Jun-11 DFB WD10.3mm 5.00kV x900 50um
C
100µm
SE 29-Jun-11 DFB WD 5.6mm 5.00kV x450 100um
D
30µm
SE 29-Jun-11 DFB WD 5.6mm 5.00kV x1.2k 30um
E
3µm
SE 28-Jun-11 DFB WD 4.9mm 5.00kV x12k 3um
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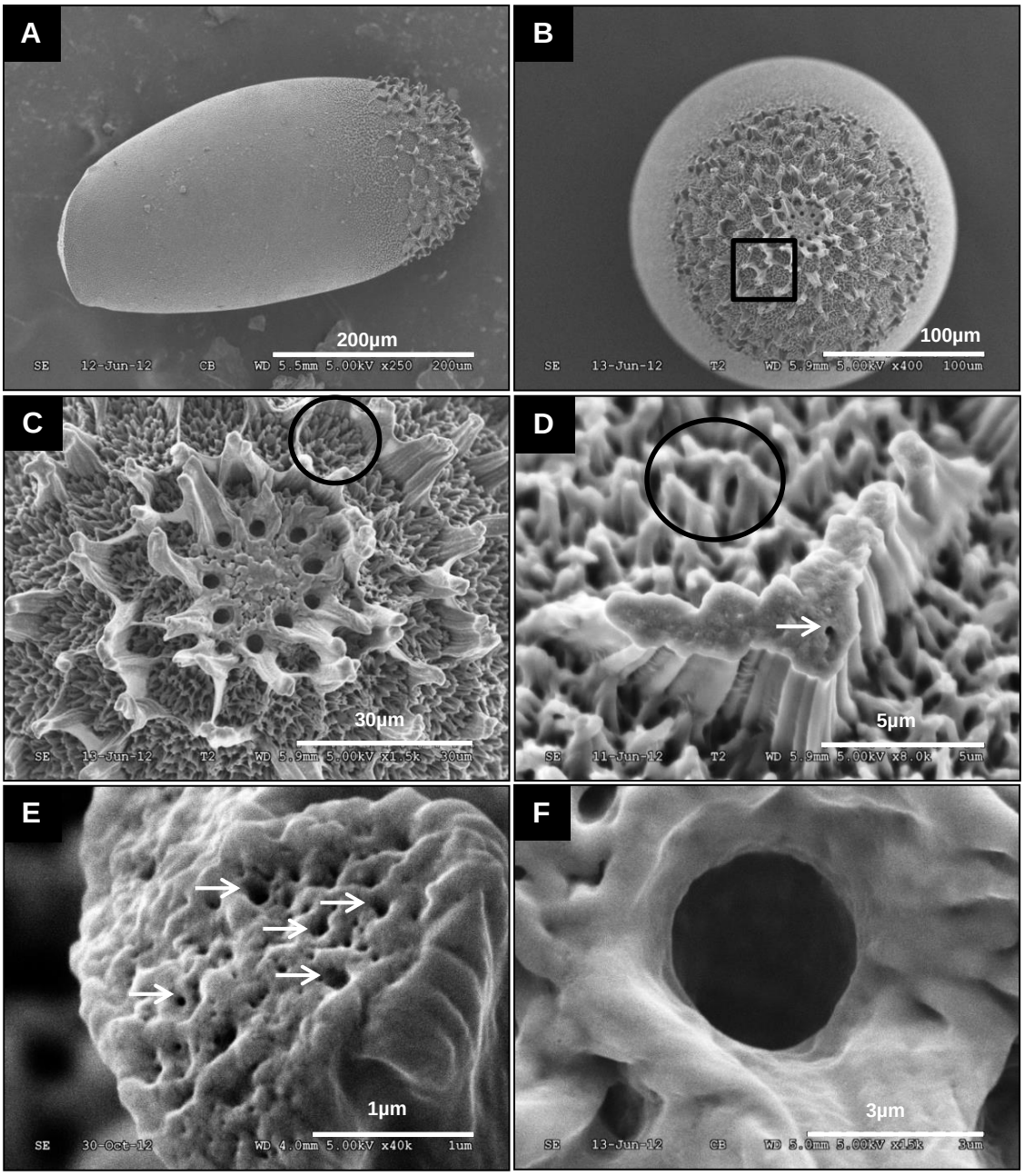
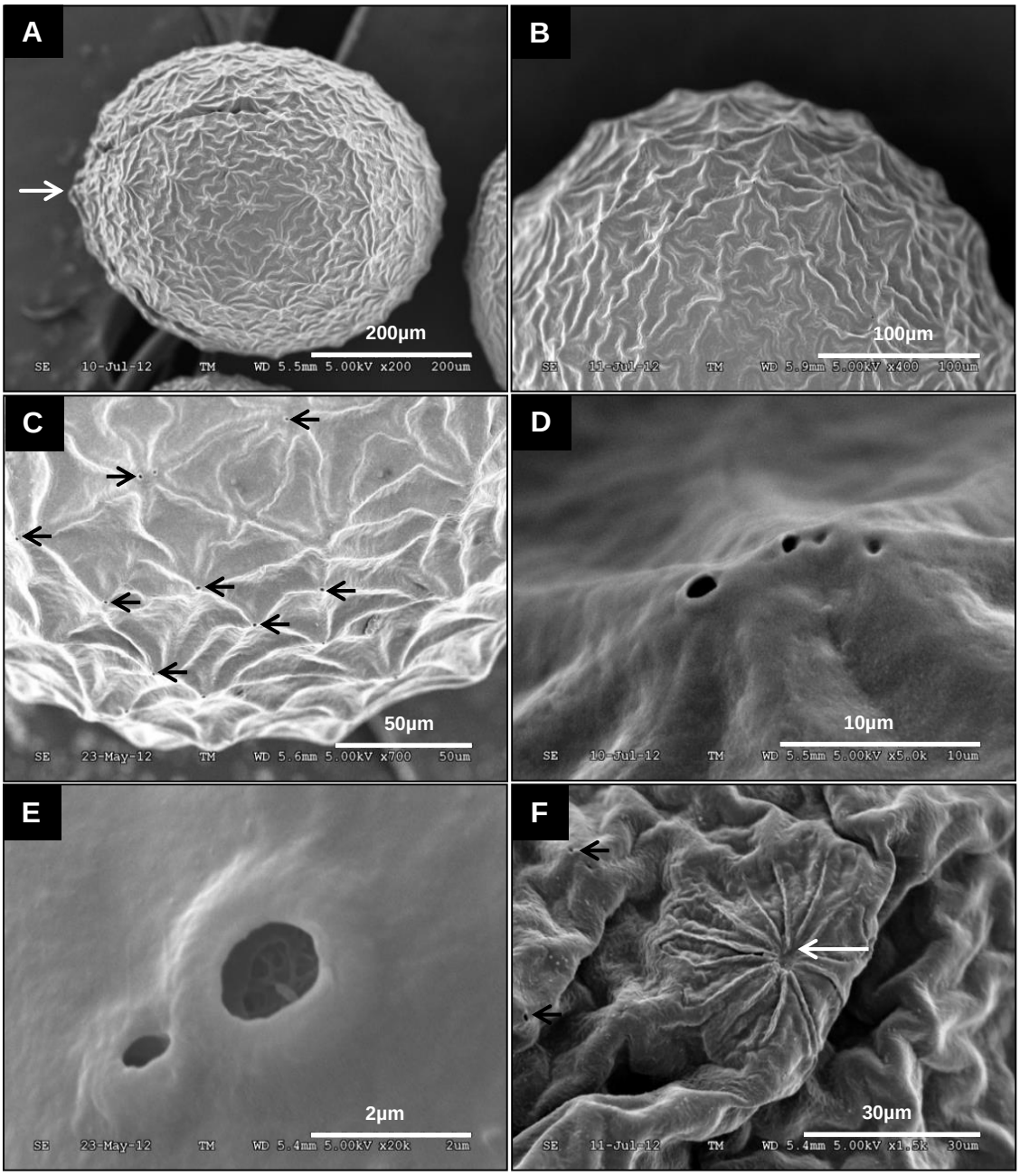
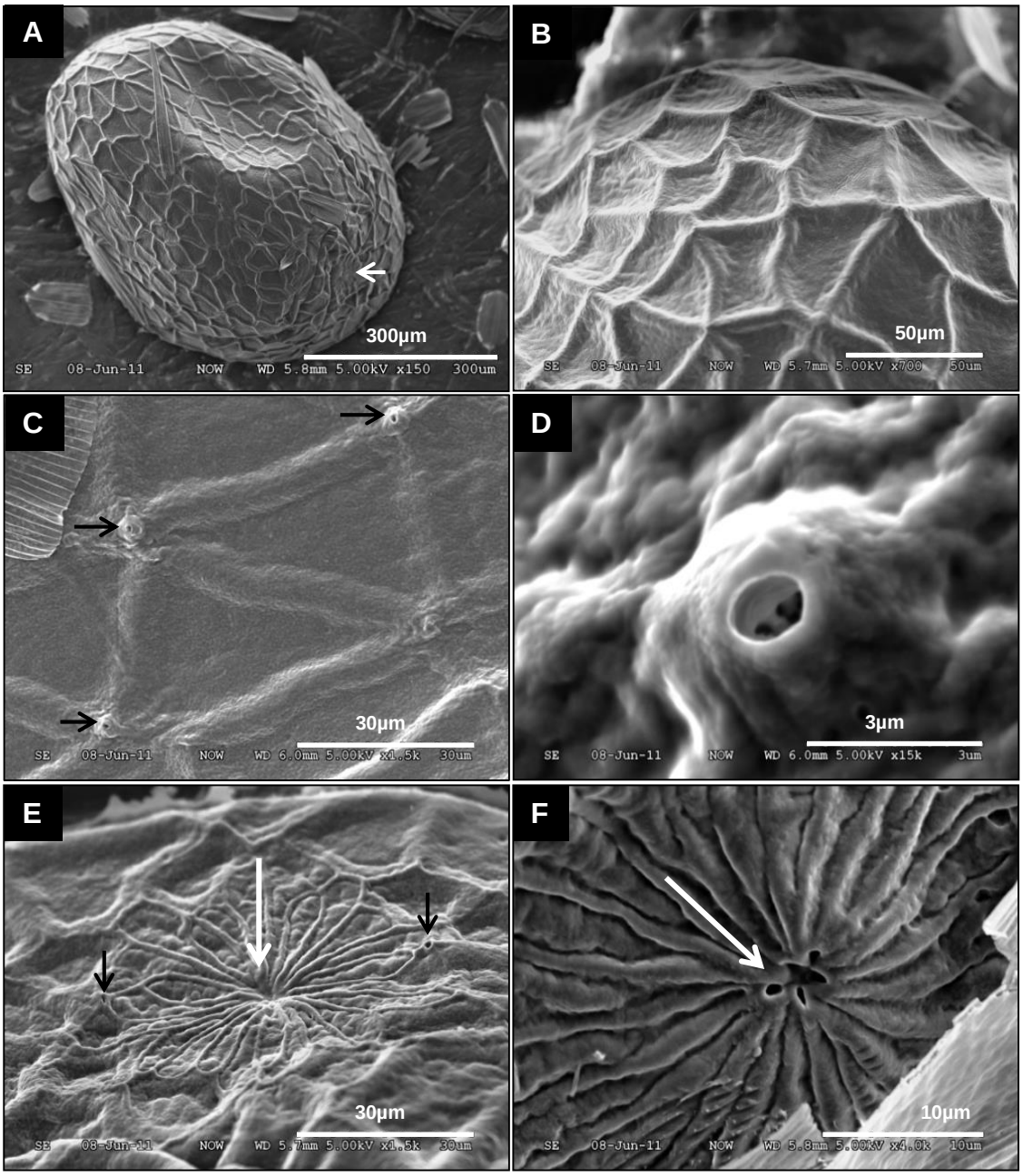
5µm
SE 14-Jun-11 DFB WD 5.5mm 15.0kV x9.0k 5um


A
200µm
SE 12-Jun-12 CB WD 5.5mm 5.00kV x250 200um
B
100µm
SE 13-Jun-12 T2 WD 5.9mm 5.00kV x400 100um
C
30µm
SE 13-Jun-12 T2 WD 5.9mm 5.00kV x1.5k 30um
D
5µm
SE 11-Jun-12 T2 WD 5.9mm 5.00kV x8.0k 5um
E
1µm
SE 30-Oct-12 WD 4.0mm 5.00kV x40k 1um
F
3µm
SE 13-Jun-12 CB WD 5.0mm 5.00kV x15k 3um


A
200µm
SE 10-Jul-12 TM WD 5.5mm 5.00kV x200 200um
B
100µm
SE 11-Jul-12 TM WD 5.9mm 5.00kV x400 100um
C
50µm
SE 23-May-12 TM WD 5.6mm 5.00kV x700 50um
D
10µm
SE 10-Jul-12 TM WD 5.5mm 5.00kV x5.0k 10um
E
2µm
SE 23-May-12 TM WD 5.4mm 5.00kV x20k 2um
F
30µm
SE 11-Jul-12 TM WD 5.4mm 5.00kV x1.5k 30um


A
300µm
SE 08-Jun-11 NOW WD 5.8mm 5.00kV x150 300um
B
50µm
SE 08-Jun-11 NOW WD 5.7mm 5.00kV x700 50um
C
30µm
SE 08-Jun-11 NOW WD 6.0mm 5.00kV x1.5k 30um
D
3µm
SE 08-Jun-11 NOW WD 6.0mm 5.00kV x15k 3um
E
30µm
SE 08-Jun-11 NOW WD 5.7mm 5.00kV x1.5k 30um
F
10µm
SE 08-Jun-11 NOW WD 5.8mm 5.00kV x4.0k 10um

CHAPTER IV

EGG MORPHOLOGY AND ULTRASTRUCTURE OF CHORIONS OF KEY STORED-PRODUCT INSECT PESTS OF U.S.A.

(To be submitted to Annals of Entomological Society of America)

Abstract. Eggs of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) were imaged with scanning electron microscopy to explore how respiratory openings on the chorion surface may be related to efficacy of fumigants. Each *P. Interpunctella* egg had many aeropyles and several micropyles; whereas each *T. castaneum* egg had neither aeropyles nor micropyles. Cross sections of chorions of *T. castaneum*, *P. interpunctella*, *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae), and *Amyelois transitella* (Lepidoptera: Pyralidae) were also imaged with transmission electron microscopy and chorion thicknesses were compared. Each species had unique chorionic structure. The types of layers and relative thicknesses of different layers of the chorion varied across species. Among the species studied, *A. transitella* had the thickest chorion. Exochorion of lepidopteran eggs was a lamellate structure and the thickest layer, whereas crystalline endochorion was the thickest layer in coleopterans. An evaluation of the feasibility of quantifying fumigant penetration into different regions of *P. interpunctella* and *C. hemipterus* eggs using osmium tetroxide as a model fumigant was also conducted. Results showed higher osmium tetroxide penetration through regions where aeropyles are localized. Further refinement of the osmium tetroxide technique developed in this study has potential to enable prediction of amounts of fumigant entering the egg via different parts of the chorion. Although quantitative data on fumigant uptake by eggs of different species are needed, the findings of the current study suggest that species-specific tolerance of eggs to fumigants may partly be explained by differences in respiratory structures and chorion characteristics among insect eggs.

Keywords: Egg respiratory system, aeropyle, fumigant efficacy, diffusion, tolerance

Insect pests pose a threat to food production, market access, food quality, and food safety. Because 9-20% of the world's food supply is destroyed or contaminated by insects after harvest (Pimental 1976), pest management programs are required and need to have a variety of control measures tailored to specific postharvest scenarios. Postharvest chamber fumigation plays a critical role when insect control is required within hours or days of harvesting to protect from infestations originating from the field or to protect stored commodities vulnerable to reinfestation by various stored-product pests. Additionally, quarantine and pre-shipment (QPS) treatment of United States-produced commodities is essential to prevent introduction of exotic pests in market countries and to maintain United States' quality standards. Fumigation with methyl bromide (MeBr), phosphine (PH_3), or sulfuryl fluoride (SF) is a favored method of insect control for United States' dried fruits and nuts, grains, processed foods, and tobacco industries. However, it has been noted that the egg stage of insects is the most fumigant tolerant stage (Bell 1976, Su and Scheffrahn 1990, Bell and Savvidou 1999, Baltaci et al. 2009, Bonjour et al. 2011, Athanassiou et al. 2012). In addition, there are variations in how eggs of different species respond to the same fumigant (Mostafa et al. 1972, UNEP 2011).

Influence of temperature, exposure time, or concentration on fumigant efficacy is well documented (Kenaga 1961, Bell 2006, UNEP 2011). Other important factors are structure and composition of the egg chorion (Outram 1967). However, very little is known about the egg morphological characters in relation to fumigant efficacy. Where a well-developed respiratory system is present, a major route of gas exchange is respiratory structures (Outram 1967). Gautam et al. (2014) compared the abundance, distribution,

and location of respiratory openings in *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae), *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae), *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae), and *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae). That study suggested a possible mechanistic relationship between respiratory openings on the chorion surface that facilitate gas exchange and the relative tolerance of insect eggs to fumigants. *C. hemipterus* eggs had only two aeropyles and no micropyle. On the other hand, eggs of *L. serricorne* and *A. transitella* had several aeropyles and many micropyles and *E. elutella* had several aeropyles and a micropyle. Interestingly, eggs of *C. hemipterus* require higher doses of SF and propylene oxide (PPO) for control compared to *L. serricorne*, *A. transitella*, and *E. elutella* (S.W., unpublished data, Su and Scheffrahn 1990, Baltaci et al. 2009, Gautam et al., unpublished data). Differences in total aeropylar and micropyler areas, external surface area, and surface to volume ratio among eggs *C. hemipterus*, *L. serricorne*, *E. elutella*, and *A. transitella* may partly explain the differences in responses of these eggs to fumigants.

Egg morphology of two key stored-product pests, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) in relation to how eggs respond to fumigants has not been investigated. Fumigant efficacy studies show that *T. castaneum* eggs are more tolerant to fumigants compared to *P. interpunctella* eggs. For example, concentrations of SF and PPO required to achieve LD_{50} for *T. castaneum* eggs were ≈5- and 3.7-times, respectively, greater than those required for *P. interpunctella* (UNEP 2011, Gautam et al., unpublished data).

In eggs with fewer or no respiratory openings, the contribution of diffusion through the chorion to the overall uptake of gases would be greater compared to eggs

with relatively larger aeropylar and micropylar areas. Tuft (1950) suggested the penetration through the chorion is 1/10 of that via the micropylar complex, an area with micropyles and aeropyles. Morphological studies of stored-product insect eggs suggest that in eggs with few or no respiratory structures, gas penetration takes place by direct diffusion through the chorion (Trougakos and Margaritis 2002, Kučerová and Stejskal 2002). Diffusion through the chorion would be a function of many factors, including physiochemical interaction between the gas and the chorion, the thickness of the chorion, the surface area of the chorion, and the surface area to volume ratio of the egg (Gautam et al. 2014). However, there is lack of information on chorion characteristics of stored-product pests. Examining the ultrastructure of chorions of different species of stored-product pests is essential to understanding choronic diffusion.

Therefore, the first objective of the current study was to compare the abundance, distribution, and location of respiratory openings in the chorions of *T. castaneum* and *P. interpunctella.* The second objective was to compare chorion structure and thickness of *C. hemipterus, T. castaneum, P. interpunctella,* and *A. transitella* to provide initial data to facilitate quantification of the consequences of egg morphology on relative tolerance of insect eggs toward fumigants. The third objective was to conduct an evaluation of the feasibility of quantifying fumigant penetration into different regions of *P. interpunctella* and *C. hemipterus* eggs using osmium tetroxide as a model fumigant.

Materials and Methods

Insects. Eggs of four species of insect pests, namely, *C. hemipterus, T. castaneum, P. interpunctella,* and *A. transitella* were used for the experiment. All eggs required for microscopy were obtained from an insectary at the United States Department

of Agriculture-Agricultural Research Service (USDA-ARS), San Joaquin Valley Agricultural Sciences Center , Parlier, CA. Voucher specimens of *C. hemipterus, T. castaneum, P. interpunctella*, and *A. transitella* adults which laid eggs used in this study were preserved in 95% ethyl alcohol and deposited at K. C. Emerson Entomology Museum at Oklahoma State University under lot numbers 138, 140, 142, and 143, respectively. Rearing conditions for all species were 27 ± 0.01°C (SE), 60 ± 0.24% RH (SE), and 16:8 (L:D) h. *Carpophilus hemipterus* were reared on ripened banana on top of soil substrate in 946-ml glass jars; *T. castaneum* on oats diet in 946-ml glass jars; and *P. interpunctella* and *A. transitella* on red flaky wheat bran diet in 3.8-liter glass jars (USDA 2007). *Carpophilus hemipterus* culture was originally obtained in 1978 from Italian Swiss Colony Winery in Fresno county, CA; *T. castaneum* in 1967 from an unknown source; *P. interpunctella* from a walnut packing house, Stanislaus Co., Modesto, CA; and *A. transitella* in 1966 from University of California, Berkeley, CA (USDA 2012).

Freshly Laid Eggs. Freshly laid eggs of *C. hemipterus* and *A. transitella* were obtained using similar procedures described by Gautam et al. (2014). Eggs were processed as described below for analysis using scanning electron microscope (SEM, S-3500N Hitachi, High Technologies America, Pleasanton, CA) or transmission electron microscope (TEM, Tecnai 12, Hillsboro, OR).

Tribolium castaneum. One hundred to 200 adults were aspirated into a 237-ml glass jar that was covered with a U.S. Standard #40 wire screen (0.42-mm openings) and secured using a metal ring. The jar containing adults was then inverted onto a 10-cm glass Petri dish lined with a filter paper and spaced (≈2-3 mm) from the filter paper using a large paper clip. Rice bran diet was placed around the edge of the Petri dish to stimulate

oviposition. The setup was then placed in a holding room with rearing conditions as described above for 2-3 d. Eggs that were 0 to 3 d old obtained on the wire screen covering the jar were processed as described below for analysis with SEM or TEM.

Plodia interpunctella. Freshly laid *P. interpunctella* eggs were collected by aspirating 100-200 adults into a 1.9-liter glass jar. The jar containing adults was covered with a 90-mm wire screen (U. S. Standard #40) (USDA 2007). The jar was inverted over an old culture jar to discard any eggs and left on its side for few minutes. The jar was then inverted on top of a large paper clip spacer, which sat on a Petri dish (90 x 20 mm) lined with a filter paper. The jar was placed in a rearing room with conditions as described above for 2-3 d. After 3 d, 0- to 3-d-old eggs were collected and processed as described below for analysis.

Scanning Electron Microscopy. *External morphology.* Freshly laid *T. castaneum* eggs were obtained attached to the wire screen. Therefore, to preserve the integrity of specimens, the wire screen containing eggs was mounted on aluminum stub using copper conductive tape with a single adhesive surface (Ted Pella, Inc., Redding, CA). Freshly collected *P. interpunctella* eggs (100-150) were mounted on double-sided carbon tabs (Ted Pella, Inc., Redding, CA) on aluminum stubs. *P. interpunctella* eggs were first attached to a piece of single-sided tape using a soft brush. Subsequently, the tape containing eggs was then attached to a double-sided sticky carbon tab on an aluminum stub with eggs exposed. To prevent specimen charging and increase secondary electron signals, eggs of all species were sputter coated with gold (SPI Module Sputter Coater) (SPI Supplies, West Chester, PA) at 5mA for 40 s at 90° to the target, followed by 40 s at 45° to the target. The samples were then viewed under a SEM and digital images were

taken at 5.00 kV. When possible, the numbers of aeropyles on exposed surfaces of individual eggs were counted. In addition, pictures of entire eggs, chorion sculpture, aeropyles, and micropyles were taken; approximately 30 to 40 pictures each of different eggs, aeropyles, and micropyles were taken for each species. Measurements made on digital images using ImageJ software (National Institute of Health, Bethesda, MD) included length and diameter (at widest point) of the egg, and diameter and cross-sectional area of each aeropyle opening. Each measurement was considered a replicate. External surface area and surface area-to-volume ratio (SA:V) were calculated. Parameters such as number of aeropyles per unit area of an egg and total aeropylar surface area were estimated, and where applicable, error was reported based on the propagation of standard errors (Lehrter and Cebrian 2010).

Cryofracturing *C. hemipterus* and *T. castaneum* Eggs. A Hitachi S-3500N (Hitachi High Technologies, America, Pleasanton, CA) scanning electron microscope equipped with a CT-1500 C cryo-unit (Quorum Technologies, East Grinstead, UK) was used to study the interior details of the chorion of freeze fractured *C. hemipterus* and *T. castaneum* eggs. Cryofracturing is a method of studying interior details of cells under a SEM by freezing the sample in slushed liquid nitrogen (a mixture of solid and liquid nitrogen) and fracturing the frozen sample while it is at liquid nitrogen temperatures (≤ -210°C) (Bozzola and Russell 1992). The eggs were aligned on a sticky tape and placed on the cryo-specimen holder, a copper stub with a groove along its diameter filled with a mixture of colloidal graphite and Tissue-Tek. The eggs were then cryo-fixed in nitrogen slush at its freezing point (≤ -210°C) for 30 seconds, transferred to the cryo-unit in the frozen state, where they were subsequently fractured, sublimated (15 min at -90°C), and

sputter coated with gold at 7 mA for (2 min). The fractured eggs were transferred to the cryostage of the SEM where they were analyzed at 15 kV and -178°C.

Transmission Electron Microscopy. Freshly laid *C. hemipterus*, *T. castaneum*, *P. interpunctella*, and *A. transitella* were prepared for transmission electron microscopy using methods based on those described in Hayat (2000). Specimens were immersed in 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.4) and held for 24 h at 4°C. After three rinses in cold buffer (sodium cacodylate buffer) specimens were post fixed overnight with 1% osmium tetroxide in the same buffer at 4°C. The specimens were then dehydrated in a graded series of ethanol (50, 70, 80, 90, 95, and 100%), 15 min each and 3 times in 100% followed by acetone. Infiltration with the "D" formulation of Spurr's resin (Spurr 1969) was done very gradually on a rotator followed by application of a gentle vacuum. Polymerization was done at 70°C overnight. Ultrathin sections (80 nm) were cut with a diamond knife on a Leica (EM UC7 Wien, Austria) Ultracut R ultramicrotome and examined with a Tecnai 12 TEM operated at 120 kV, and imaged with a SIS (Lakewood, CO) Megaview III camera. The purpose of transmission electron microscopy was to study the ultrastructure of the different chorions. Approximately 30-40 images of the cross section of chorion of each species were taken. Measurements made on TEM images using ImageJ software (National Institute of Health, Bethesda, MD) included thickness of the different layers of the chorion. Each measurement was considered a replicate.

Penetration of Osmium Tetroxide into *Carpophilus hemipterus* and *Plodia interpunctella* Eggs. Light microscopy was used for evaluating penetration of osmium tetroxide into *C. hemipterus* and *P. interpunctella* eggs. These two species were selected

for evaluation because each *C. hemipterus* egg only has only two aeropyles localized at the anterior end, whereas eggs of *P. interpunctella* have several aeropyles that are localized at both ends of each egg. Freshly laid eggs were mounted on plastic coverslips (22 x 22 x 0.18 mm) (Ted Pella, Inc., Redding CA) that had previously been cut into strips (7 x 22 mm). The plastic strips were coated with Duro-Tak 387-2287/87-2287 (National Starch & Chemical Co., Westchester, IL). After mounting, the eggs were viewed with a stereoscope (Leica M2 125, Leica Microsystems, Buffalo Groove, IL) and pictures of eggs before fumigation with osmium tetroxide were taken (untreated control) using a Spot Insight 2 digital camera interfaced with a PC using Spot Basic software (Diagnostic Instruments, Inc., Sterling Hights, MI). The eggs were then exposed to osmium tetroxide vapor for 1 h. The apparatus used for exposing eggs to osmium tetroxide vapor was assembled by screwing two 4-ml vials with 13-425 caps (224952, Wheaton Micro Kit) onto a connecting adapter with 13-425 screw threads (WF033112, Wheaton Micro Kit) using WHEATON Connection®, 13-425 to 13-425. A connecting adapter had three arms; opposite sides of which were screwed two 4-ml vials and a third arm that was a modified stop valve. Osmium tetroxide crystal was placed in one of the 4-ml vial. The WHEATON Connection® that connected the vial with osmium tetroxide to the adapter also had a modified stop valve to prevent leakage of osmium tetroxide vapor when not in use. An osmium tetroxide crystal was placed in one of the 4-ml vials for 24 h, with the stop valve closed, to develop a concentration of ≈10,000 ppm. Plastic strips with eggs were then placed in the other 4-ml vial and screwed to the adapter. Subsequently, the stop valve of the vial that contained osmium tetroxide crystal was opened and the air containing osmium tetroxide vapor was pumped into the apparatus

using a 5-ml gas syringe (Precision syringe, Dynatech Precision Sampling, Baton Rouge, LA) from the stop valve at the third arm of the adapter. After 1 h, the eggs were aerated for 10-15 min. Then, the strips of fumigated eggs were observed under a Leica M2 125 stereoscope and digital pictures were taken using a Spot Insight 2 digital camera interfaced with a PC using Spot Basic software. Images of control and fumigated eggs were analyzed.

Statistical Analysis. To test the null hypothesis that the external surface areas and surface-to-volume ratios of eggs, and chorion, exochorion, and endochorion thicknesses were equivalent across insect species, an analysis of variance (ANOVA) was conducted for each parameter using Statistical Analysis System software (SAS Institute 2010) and the PROC GLM model. If the null hypothesis was rejected, the ANOVA was followed by Tukey HSD comparison tests to determine if means were statistically different (alpha = 0.05).

Results

General Egg Morphology. *Tribolium castaneum.* Eggs are ovate in shape (Fig. 1A-C), 381.3-710.8 µm long, and 290.7-324.4 µm in diameter at the widest point (Table 1). Both anterior (narrower) and posterior (broader) ends are bluntly rounded (Fig. 1B-E). The surface of the chorion is smooth under low magnification (100-180x) (Fig. 1B-D). However, at higher magnifications (>2,500x), microscopic threads were observed on the surface of the chorion (Fig. 1F), and this was more pronounced at the posterior end. In ≈20% of the eggs observed, there was what appeared to be a cluster of microscopic threads on the posterior end (Fig. 1C – indicated by a white arrow). No aeropyles or micropyles were observed on the surface of the chorion. Interestingly, in images of *T.*

castaneum eggs taken using a Leica M2 125 stereoscope, 10% of the entire volume of the egg on one end was clear and appeared to be filled with gas or liquid (Fig. 1A – indicated within the white circle).

Plodia interpunctella. Eggs are ovate in shape (Fig. 2A), 347.2-433.2 µm long, and 179.1-247.5 µm in diameter at the widest point (Table 1). The chorion is marked by numerous tubercle-like winding ridges that were joined at termini but did not have an angular pattern (Fig. 2A-D). One or two aeropyles were present at each terminus (Fig. 2C-E). Aeropyles were distinctly characterized by a broad collar (Fig. 2E). Collar is a thickened and raised chorion forming the rim of an aeropyle (Arbogast et. al 1980). Termini were distributed over the entire egg surface, but those with aeropyles were localized near the ends (Fig. 2B and C). At a high magnification (9,000x), the aeropyle opens to an inner chorionic meshwork (Fig. 2E). Approximately 10% of the observed eggs had a nipple-like projection formed by chorionic folds at the tip of the anterior end (Fig. 2A - indicated by a white arrow). A micropylar area was located at the anterior end (Fig. 2A and B – indicated by a white arrow). Four to five micropyles were found at the center of the micropylar rosette that was formed by 19-26 primary cells (Fig. 2F – indicated by a white arrow). The primary cells are distinct loop-like or petal-like structures that have distinct ridges or carinae. Radiating out from the central depression at the apical end, they give a rosette appearance to the micropylar region (Baker et al. 2012).

External Surface Area and Volume. External surface areas and SA:V ratios of *T. castaneum* and *P. interpunctella* were not similar (F =54.9; df = 1,58; $P < 0.0001$ and F =52.2; df = 1,58; $P < 0.0001$, respectively; Table 1). The average external surface area

of a *T. castaneum* egg was significantly larger relative to a *P. interpunctella* egg (Table 1). However, *P. interpunctella* had greater SA:V ratio compared to *T. castaneum* (Table 1).

Respiratory Structures. *Aeropyles. T. castaneum* egg did not have any aeropyles, hence aeropylar parameters of *T. castaneum* were not calculated. *P. interpunctella* had 13.6 ± 0.25 (mean ± SE) aeropyles per egg and each aeropyle opening was 2.39 ± 0.11 μm (mean ± SE) in diameter (Table 1). The aeropyles were localized at the ends and apparently none were found near the mid-section (Table 1). The numbers of aeropyles per square micrometer in *P. interpunctella*, estimated by dividing the average number of aeropyles per egg by the average external surface area of an egg was $5.2 \times 10^{-5} \pm 1.9 \times 10^{-7}$ (mean ± SE) (Table 1). The combined area of all the aeropyle openings per egg, the total aeropylar surface area, was 52.1 ± 4.3 μm^2 (mean ± SE) based on estimation by multiplying the average cross-sectional area of an aeropyle and the average number of aeropyles per egg (Table 1).

Micropyles. The micropylar surface area to route gas exchange was negligible (<1%) in *P. interpunctella*, and *T. castaneum* egg did not have any micropyle.

General Chorion Morphology. *C. hemipterus.* Freeze-fractured SEM images of a *C. hemipterus* egg showed that it is outlined by a continuous chorion (Fig. 3A). Intrachorionic meshwork of variable thickness was observed underneath the chorion (Fig. 3B and C). This intrachorionic meshwork held intrachorionic air spaces between pillars of the meshwork (Fig. 3). The TEM images show that *C. hemipterus* chorion is a 4-layered structure and the thicknesses of the layers vary (Fig. 4A). The first outer layer is a discontinuous mucous layer (0.045-0.075 μm) that surrounds the egg (Fig. 4B and C).

Beneath the mucous layer is a very thin <0.02 µm membrane which is followed by a continuous 0.21-0.45 µm exochorion. The endochorion is a multilayered crystalline structure that is 2.25-3.71 µm and is lined from inside with the vitelline membrane. The overall thickness range of the *C. hemipterus* egg chorion is 2.70-4.23 µm.

T. castaneum. Freeze-fractured SEM images of *T. castaneum* egg showed that a continuous chorion layer covers the egg (Fig. 5A). Beneath the chorion, intrachorionic air spaces are held between the pillars of intrachorionic meshwork. This meshwork was of variable thickness (Fig. 5B). A vitelline membrane of variable thickness was either directly attached to the chorion or was present after the intrachorionic meshwork (Fig. 5C). TEM images show that the *T. castaneum* chorion is a 5-layered structure (Fig. 6A-C). The outermost layer is 0.11-0.62 µm mucous layer. Beneath the outermost mucous layer is a continuous 0.25-0.95 µm exochorion (Fig. 6B and C). This layer is followed by a low electron density 0.22- to 0.62-µm mesochorion. The fourth layer of the chorion is the 0.35- to 0.58-µm thick endochorion. This layer is followed by a second endochorion layer which is a multilayered crystalline structure that is 1.30-2.02 µm in thickness. The overall thickness range of the *T. castaneum* chorion is 2.01-4.34 µm.

P. interpunctella. TEM images of *P. interpunctella* chorion show that it is composed of four distinct layers (Fig. 7A-C). The outermost layer is a mucous layer that is 0.08-0.81 µm. A membrane which is <0.020 µm thick is covered by the mucous layer. Beneath the membrane is a 0.86- to 2.14-µm thick lamellate exochorion. The thickness of the exochorion is extended at the regions where ridges occur and at the termini (Fig. 7C). The third layer of the chorion is a 0.20- to 0.81-µm trabecular layer sustained by pillars branching on its base and at the top. The thickness of the trabecular layer was variable

and extended at the ridges (Fig. 7B and C). The endochorion is very thin layer that is 0.06-0.20 µm and is found between trabecular layer and the vitelline membrane. The overall thickness range of the *P. interpunctella* chorion was 1.18-4.11 µm.

A. transitella. TEM images of *A. transitella* chorion show that it is composed of five distinct layers (Fig. 8A-C). The outermost layer is a thick mucous layer of variable thickness ranging from 0.26-3.43 µm (Fig. 8A-C). Underneath the mucous layer is a <0.020 µm membrane (Fig. 8A-C). A thin wax layer may be present covering the mucous layer (Fig. 8B). Beneath the membrane is a 1.33- to 4.11-µm thick lamellate exochorion. The thickness of the exochorion is extended at the regions where ridges are present and at the termini (Fig. 8B and C). The third layer of the chorion is a 0.20- to 0.81-µm trabecular layer sustained by pillars branching on its base and at the top. The thickness of the trabecular layer was variable and extended at the ridges (Fig. 8C). The endochorion is a very thin 0.06- to 0.56-µm layer and is found in between trabecular layer and the vitelline membrane. The overall thickness range of the *A. transitella* chorion was 2.06-7.56 µm.

Chorion thickness. Chorion thickness varied across the species tested (F =24.8; df = 3,116; $P < 0.0001$). Among the species tested, *A. transitella* had the thickest chorion (Table 2). The chorions of *C. hemipterus* and *T. castaneum* had similar thicknesses and were greater than that of *P. interpunctella* (Table 2). Similarly, thicknesses of the exochorion and endochorion also varied across species (F =113.9; df = 3,116; $P < 0.0001$ and F =975.0; df = 3,116; $P < 0.0001$). *A. transitella* had the thickest exochorion. Exochorion of *P. interpunctella* was thicker than exochorions of *C. hemipterus* and *T. castaneum* (Table 2). For endochorions, thickness was greatest in *C. hemipterus* followed

by *T. castaneum*. Thicknesses of *P. interpunctella* and *A. transitella* endochorions were not significantly different (Table 2).

Penetration of Osmium Tetroxide into *Plodia interpunctella* and *Carpophilus hemipterus* Eggs. *P. interpunctella* and *C. hemipterus* eggs fumigated with osmium tetroxide change in color from cloudy white to brown or black as a result of osmium teroxide reacting with unsaturated double bonds in lipids and proteins (Fig. 9; Bozzola and Russel 1992). Black is found in areas with high concentrations of osmium tetroxide. Treated *P. interpunctella* eggs show black color at the anterior and posterior poles where aeropyles are present (Fig .9B and E), whereas untreated eggs are uniformly cloudy white (Fig. 9A). Mid regions of *P. interpunctella* eggs were of light-brown color but different than that in the control eggs. *C. hemipterus* eggs showed changes in color at the tips (Fig. 9E).

Discussion

The current study provides quantitative information on egg morphological and physical factors that are likely to influence gas exchange in eggs of two key stored-product insect pests, namely, *T. castaneum* and *P. interpunctella*. These factors for each species are: the number of aeropyles present, aeropylar area, external surface area, the percentage of external surface area covered by chorion, and estimated egg volume. Additionally, structural differences among the layers of the chorions and differences in thicknesses of the chorionic layers among *C. hemipterus*, *T. castaneum*, *P. interpunctella*, and *A. transitella* are compared. Information on cross sections of freeze fractured *C. hemipterus* and *T. castaneum* eggs is provided. Lastly, the potential of using osmium tetroxide as a model fumigant molecule for quantification of gas entry into stored-product

insect eggs via various routes such as the chorion, aeropyles, and micropyles was explored. This is an initial but critical step in demonstrating higher gas penetration through areas of the egg with aeropyles than by direct diffusion through the chorion.

Egg morphology of *P. interpunctella* is generally consistent with the description by Arbogast et al. (1980). External morphology features of *P. interpunctella* eggs are similar to those of other lepidopteran eggs, namely, *E. elutella* and *A. transitella* described by Gautam et al. (2014). Each *P. interpunctella* egg has ≈14 aeropyles, whereas *A. transitella* and *E. elutella* eggs have ≈8 and 18 aeropyles each, respectively (Gautam et al. 2014). In comparison to *A. transitella* and *E. elutella*, *P. interpunctella* has wider aeropyle openings, hence comparatively larger aeropylar area. Aeropylar area, of *P. interpunctella* is 2.5- and 9.5-times larger than that of *E. elutella* and *A. transitella*, respectively. Aeropylar area of *P. interpunctella* is 24.1-times larger - than that of *C. hemipterus*, a coleopteran species with eggs that have only two aeropyles (Gautam et al. 2014). The average number of aeropyles and total aeropylar area in *L. serricorne* were ≈21,000 and 19-times, respectively, greater than in eggs of *P. interpunctella*. Respiratory openings were not found on the chorion of *T. castaneum* eggs. There are currently no published SEM and TEM descriptions of species belonging to family Tenebrionidae, which includes *T. castaneum*. Interestingly, the numerous intrachorionic air spaces observed in *T. castaneum* suggest that appreciable volumes of gas may be held in these spaces. A majority of terrestrial insect eggs have gas-filled meshwork in the chorion (Hinton 1981). The introduction of gas into the chorion may be happening when the egg is still inside the mother's reproductive system (Wigglesworth and Beament 1950). The gas-filled intrachorionic meshwork is connected to the environment via aeropyles that are

on the surface of the chorion (Tuft 1950, Hinton 1981). In *C. hemipterus* and *T. castaneum* eggs that have only two and no aeropyles, respectively, the intrachorionic meshwork is extensive and suggests that eggs may hold larger volumes of gas compared to *E. elutella*, *A. transitella*, and *P. interpunctella* eggs with less extensive meshwork and several aeropyles and micropyles.

Based on data from the current study and that by Gautam et al. (2014), the average diameters of the openings of aeropyles for all the species studied ranged from 0.1-2.39 µm. This is at least three orders of magnitude greater than the size of O_2 (1.2 Å), MeBr (2.49 Å), SF (2.99 Å), PH_3 (2.05Å), ozone (O_3) (1.42 Å), and PPO (2.11 Å), and are expected to easily accommodate the mean free path of gas molecules (1000 Å) (Hinton 1963) under conventional fumigation conditions. Even though contribution of respiratory versus chorionic diffusion is not directly probed, it could be argued that diffusion through respiratory structures is greater than direct diffusion through the chorion. Daniel and Smith (1994) demonstrated that the shape and size of the egg pore, a respiratory structure on the surface of the egg, influences gas uptake by an egg. Comparison of respiration rates using Gibson respirometer showed that in the egg that had 2-fold larger aeropyle opening, gas exchange was 2-3 fold greater. The structural compositions of the chorions of four species studied were unique for each species. *P. interpunctella* and *A. transitella* had a lamellate exochorions, a characteristic feature in lepidopteran eggs. Similar chorion characteristics for other lepidopteran species are abundant in literature (Fehrenbach et al. 1987, Cônsoli et al. 1999). *C. hemipterus* and *T. castaneum* had crystalline endochorion, which is a characteristic feature of coleopteran eggs (Furneaux and Mackay 1972). Fumigant susceptible eggs of lepidopteran species *P.*

interpunctella and *A. transitella* eggs had a trabecular layer beneath the exochorion, but the relatively fumigant tolerant eggs of *T. castaneum* and *C. hemipterus* did not have the trabecular layer. The endochorion layers of *T. castaneum* and *C. hemipterus* were ≈20-fold thicker than the endochorion layers of *P. interpunctella* and *A. transitella*. Structural proteins present in the chorions of coleopteran and lepidopteran species may be different. Outram suggests that structural proteins in egg chorions and embryonic membranes may bind to fumigant molecules and affect their efficacy (Outram 1967). Therefore, the function of the trabecular layer in fumigant-susceptible *P. interpunctella* and *A. transitella* eggs and the function of endochorionic layer in fumigant-tolerant *T. castaneum* and *C. hemipterus* eggs need to be further investigated from a fumigant efficacy point of view.

Investigation of the relative contribution of chorionic diffusion and respiratory structure-mediated gas uptake using osmium tetroxide is critical to understanding how egg morphology could be related to the relative tolerance of insect eggs to fumigants. Diffusion through the micropylar region in *Rhodnius* eggs was ≈10-times greater than chorionic diffusion (Tuft 1950). In an experiment to study distribution of air within the insect egg shell by cobalt sulphide injection technique, Wigglesworth and Beament (1950) demonstrated that air is confined to an inner layer of the porous chorion that is connected to the atmosphere via aeropyles. In the current study, the light microscopy images of *P. interpunctella* and *C. hemipterus* eggs taken before and after fumigation using osmium tetroxide seem to indicate relatively easier movement of gases into the egg via aeropyles than the chorion. X-ray detection performed with a Evex QDD Violin detector (Evex Analytical Instruments, Inc, Princeto, NJ) using either line or multipoint

analysis routines (NanoAnalysis software, Evex Analytical Instruments, Inc, Princeton, NJ) on the longitudinal sections of *C. hemipterus* and *P. interpunctella* eggs showed higher concentrations of osmium near the poles than at the center of the egg (unpublished data). This may suggest greater gas penetration via aeropyles than through the chorion.

Despite the fact that the relative efficacy of fumigants as a function of respiratory structures on the surface of the chorion has not been directly probed, critical insight into this relationship can be extracted from literature. In general, coleopteran eggs require higher concentrations of SF and PPO to control compared to lepidopteran eggs; eggs of *L. serricorne* are the exception (Su and Scheffrahn 1990, Baltaci et al. 2009, Gautam et al. unpublished data, S.W., unpublished data). Data from the current study and that by Gautam et al. (2014) suggest that egg morphological factors such as number of aeropyles present, aeropylar area, and surface to volume ratio may directly influence fumigant penetration or uptake. These data make it logical to postulate that there may be greater gas penetration via aeropyles than through the chorion. In addition, the structural composition and the thickness of the chorion may influence gas penetration through the chorion.

A possible link between the respiratory mechanism of an egg and fumigant efficacy can be hypothesized. The fact that *C. hemipterus* and *T. castaneum* eggs had few or no aeropyles, but a greater intrachorionic meshwork may suggest reduced gas exchange in these two species compared to lepidopteran species that have relatively thinner intrachorionic meshwork but a greater number of aeropyles. The findings of this study are a critical initial step in systematically exploring the role of egg morphology and molecular diffusivity in the context of species-specific fumigant efficacies. Future

research will be aimed at refining microscopic and molecular marker techniques to elucidate species specific ovicidal deficiencies of fumigants.

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Table 1. Parameters (mean ± SE) and descriptions of eggs and location, abundance, and attributes of respiratory openings of *T. castaneum* and *P. interpunctella* eggs (*n* = 30).

Attribute	*T. castaneum*	*P. interpunctella*
Length (µm) (Range)	553.4 ± 17.3 (381.3-710.8)	443.4 ± 4.4 (347.2-433.2)
Diameter (µm) (Range)	307.6 ± 1.8 (290.7-324.4)	291.4 ± 2.0 (179.1-247.5)
L/W ratio	1.79 ± 0.57	1.52 ± 0.02
Surface area (μm^2)*	$3.4 \times 10^5 \pm 9.9 \times 10^3$a	$2.6 \times 10^5 \pm 3.4 \times 10^3$b
Surface to volume ratio	$24.9 \times 10^{-3} \pm 1.6 \times 10^{-4}$b	$26.8 \times 10^{-3} \pm 1.7 \times 10^{-4}$a
Surface of the chorion	Smooth; microscopic threads at tips	Tubercles joined together by ridges at termini
Aeropyles n = 40	Absent **	Present; 13.6 ± 0.25 Distributed at ends;
Diameter of aeropyle (µm)	N/A	2.39 ± 0.11
Cross sectional area of aeropyles (μm^2)	N/A	3.83 ± 0.31
Number of aeropyles per μm^2 of an egg	N/A	$5.2 \times 10^{-5} \pm 1.9 \times 10^{-7}$
Total aeropylar surface area per egg (μm^2)	N/A	52.1 ± 4.3
Micropyles	Absent	Present; 4-5 micropyles

*Means within a row followed by different letters are significantly different ($\alpha = 0.05$).

***T. castaneum* did not have any aeropyles, hence aeropylar parameters are not calculated.

Table 2. Chorion, exochorion, and endochorion thicknesses (mean ± SE) for *C. hemipterus, T. castaneum, P. interpunctella,* and *A. transitella* eggs (*n* = 30).

Insect species	Chorion thickness (µm)	Exochorion thickness (µm)	Endochorion thickness (µm)
C. hemipterus	3.29 ± 0.08b	0.31 ± 0.01c	3.01 ± 0.08a
T. castaneum	3.22 ± 0.12b	0.44 ± 0.03c	2.01 ± 0.14b
P. interpunctella	2.12 ± 0.14c	1.31 ± 0.06b	0.11 ± 0.01c
A. transitella	4.02 ± 0.24a	2.12 ± 0.14a	0.15 ± 0.02c

[a]Means within a column followed by different letters are significantly different (α = 0.05).

Figure Caption

Fig. 1. *T. castaneum* egg. Light microscope image showing a clear region embedded within the chorion (indicated by a circle) (A), scanning electron micrograph of an egg (100x) (B), egg showing extended layer of the chorion at the posterior end (indicated by an arrow) (150x) (C), view of an egg from the top of anterior end (180x) (D), sculpture of the chorion (600x) (E), and a magnified image of the chorion sculpture at the posterior end showing micro threads (2,500x) (F).

Fig. 2. Scanning electron micrograph of a *P. interpunctella* egg (200x) (A), sculpture of the anterior-end chorion with micropyler area indicated by a white arrow and aeropyles indicated by black arrows (400x) (B), the posterior end of the egg with aeropyles indicated by black arrows (500x) (C), aeropyles atop termini (1,500x) (D), a magnified aeropyle showing the inner chorionic meshwork (9,000x) (E), and the anterior end of the egg with micropyles indicated by a white arrow (2,000x) (F).

Fig. 3. Scanning electron micrograph of a freeze-fractured *C. hemipterus* egg. General view of the ultrastructure of a *C. hemipterus* egg (4,000x) (A), cross section of the egg showing chorion (ch), intrachorionic meshwork (icm), and serosal cuticle (sc) (9,000x) (B), cross section showing extended intrachorionic meshwork and intrachorionic air space (1,800x) (C).

Fig. 4. Transmission electron micrographs of *C. hemipterus* egg. General view of the ultrastructure of the egg (A), cross section of the chorion showing different layers and relative thicknesses (B), cross section of the chorion showing the mucous layer (C) (ml=mucous layer, m=membrane, exo=exochorion, end=endochorion, vm=vitelline membrane, icm=intrachoronic meshwork, ics=intrachorionic air space).

Fig. 5. Scanning electron micrographs of a freeze-fractured *T. castaneum* egg. General view of the ultrastructure of *T. castaneum* egg (2,000x) (A), cross section of the egg showing chorion (ch), intrachorionic meshwork (icm), and intrachorionic air space (ics) (9,000x) (B), cross section showing chorion and vitelline membrane (vm) (1,800x) (C).

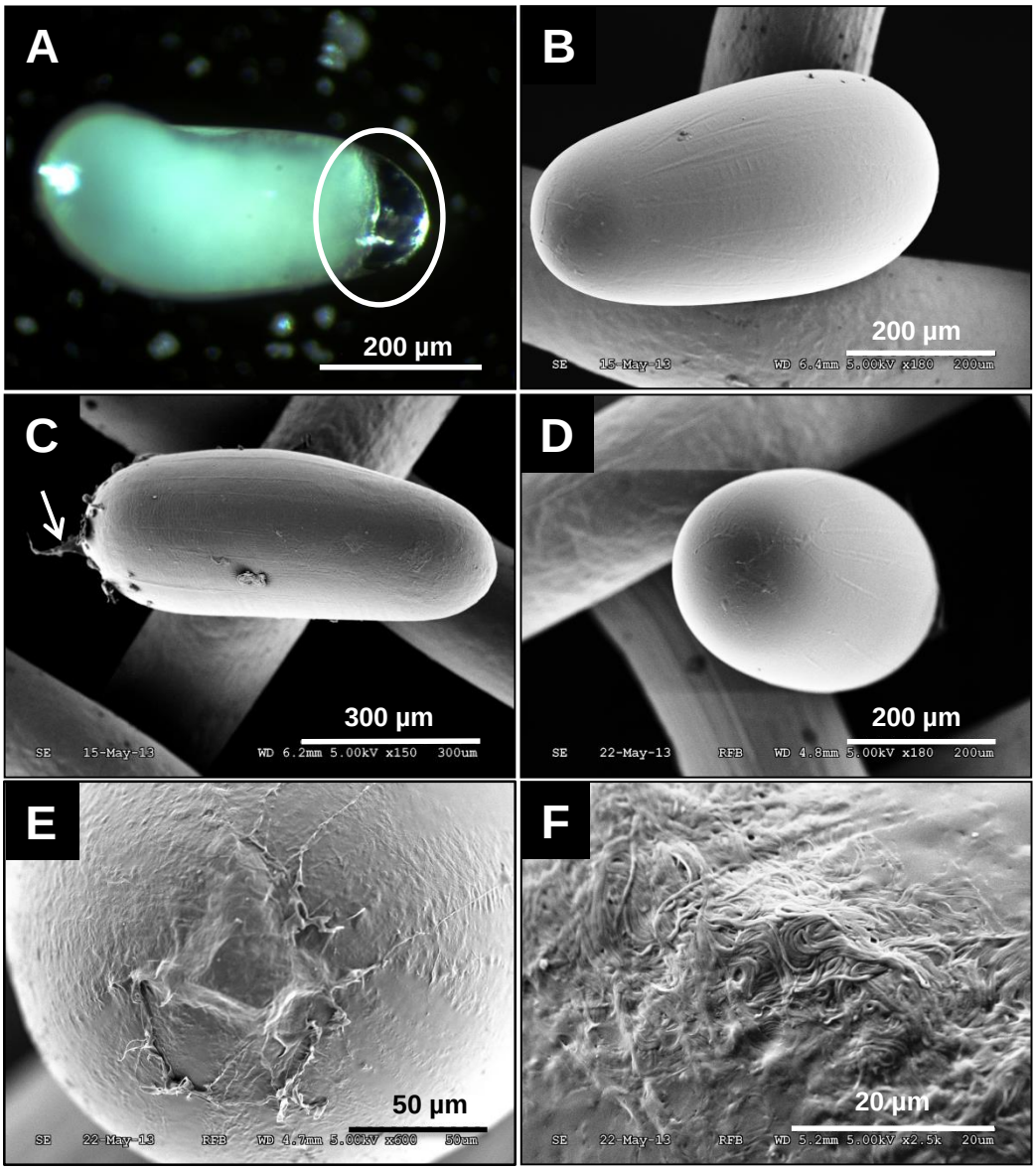
Fig. 6. Transmission electron micrographs of *T. castaneum* egg. General view of the ultrastructure of the egg (A), cross section of the chorion showing different layers and relative thicknesses (B), cross section of the chorion showing mucous layer (C) (ml=mucous layer, exo=exochorion, me=mesochorion, end1= first endochorion layer, end2=second endochorion layer, vm=vitelline membrane, ics=intrachorionic air space).

Fig. 7. Transmission electron micrographs of *P. interpunctella* egg. General view of the ultrastructure of the egg (A), cross section of the chorion showing different layers and relative thicknesses (B), cross section of the chorion extended exochorion at the termini (C) (ml=mucous layer, m=membrane, exo=exochorion, tr=trabecular layer, end=endochorion, vm=vitelline membrane).

Fig. 8. Transmission electron micrographs of *A. transitella* egg. General view of the ultrastructure of the chorion (A), cross section of the chorion showing different layers and relative thicknesses (B), cross section of the chorion showing wax layer (C) (wl=wax layer, ml=mucous layer, m=membrane, exo=exochorion, tr=trabecular layer, end=endochorion, vm=vitelline membrane).

Fig. 9. Light microscopy images of *P. interpunctella* and *C. hemipterus* eggs. *P. interpunctella* eggs before fumigation with osmium tetroxide (80x) (A), after 1-h fumigation with osmium tetroxide showing brown middle regions and black regions at the poles (80x) (B), *C. hemipterus* eggs before fumigation with osmium tetroxide (80x)

(C), and after fumigation with osmium tetroxide showing brown regions and a black region at the anterior pole with aeropyles (80x) (D), a *P. interpunctella* egg with black regions at the poles (160x) (E), a *C. hemipterus* egg with a black tip indicated by a black arrow and circle.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E
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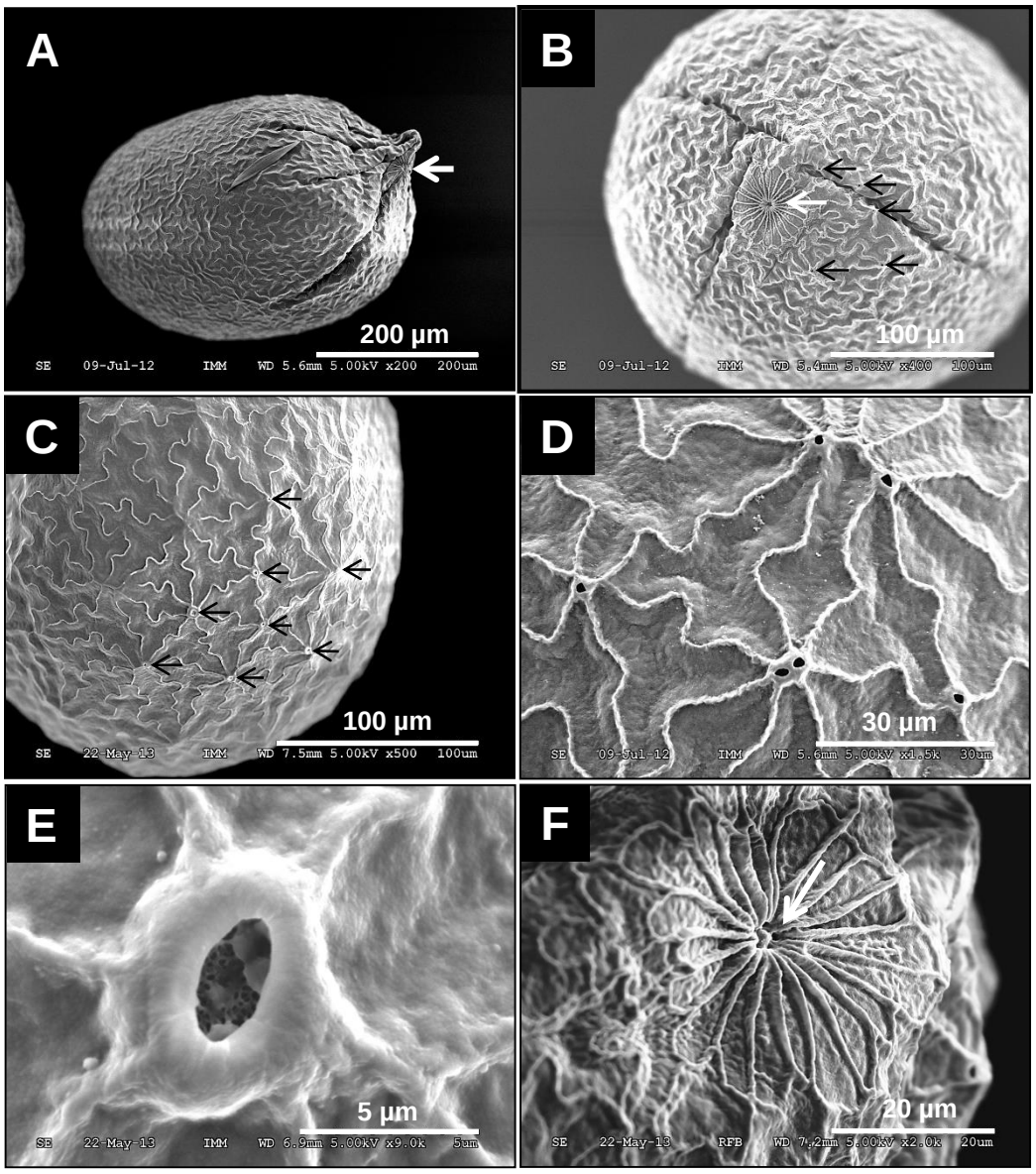
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E
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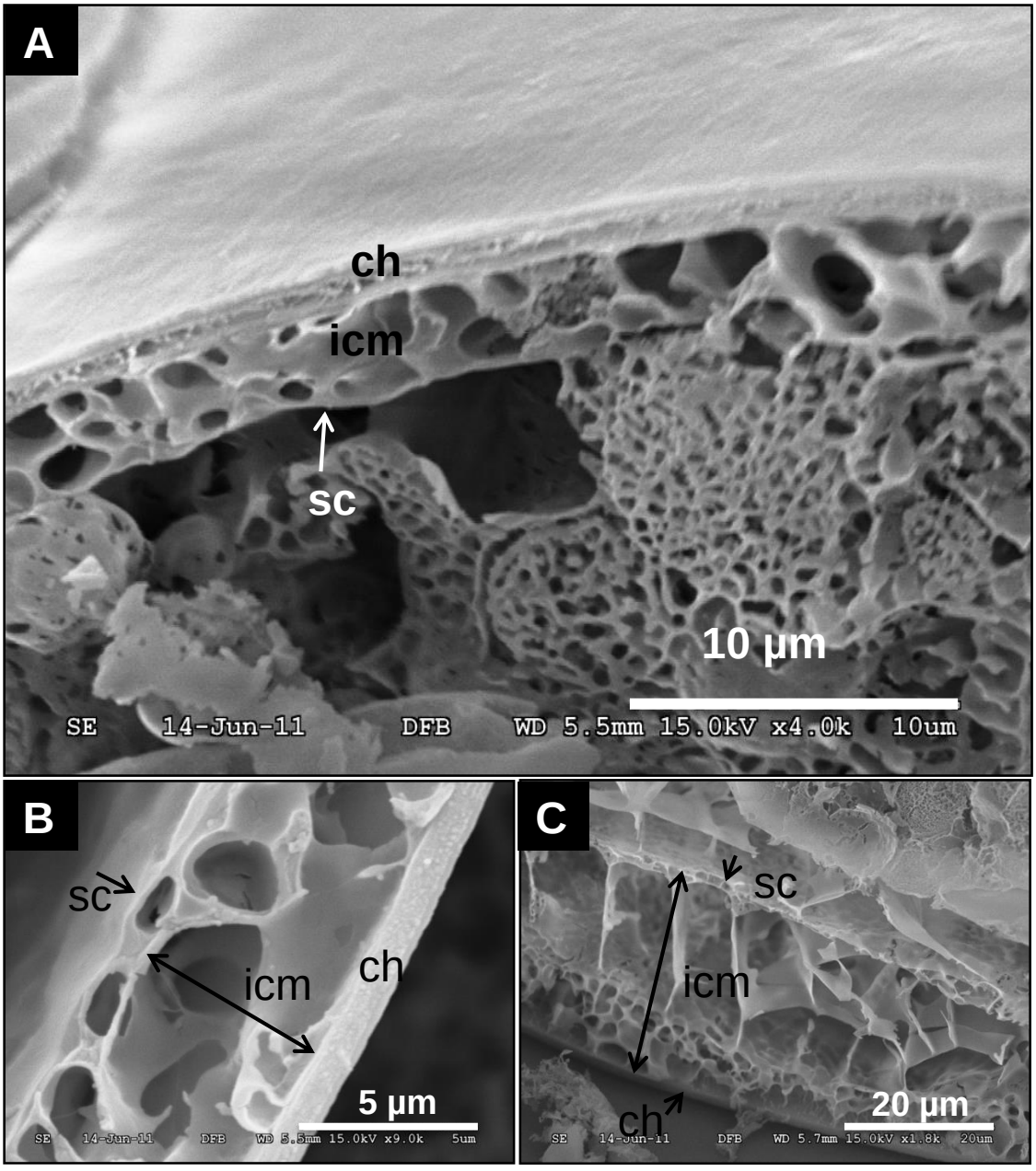
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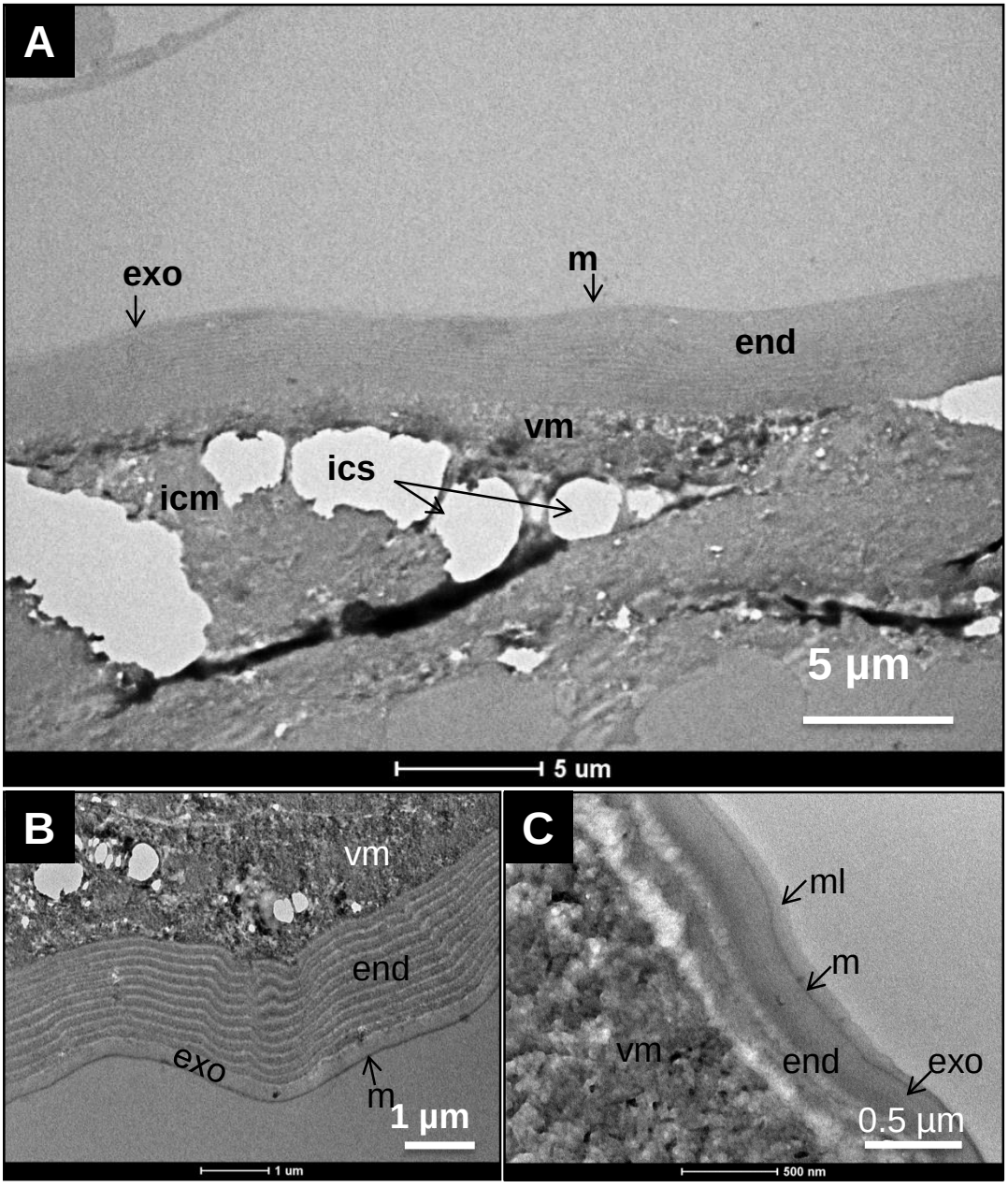
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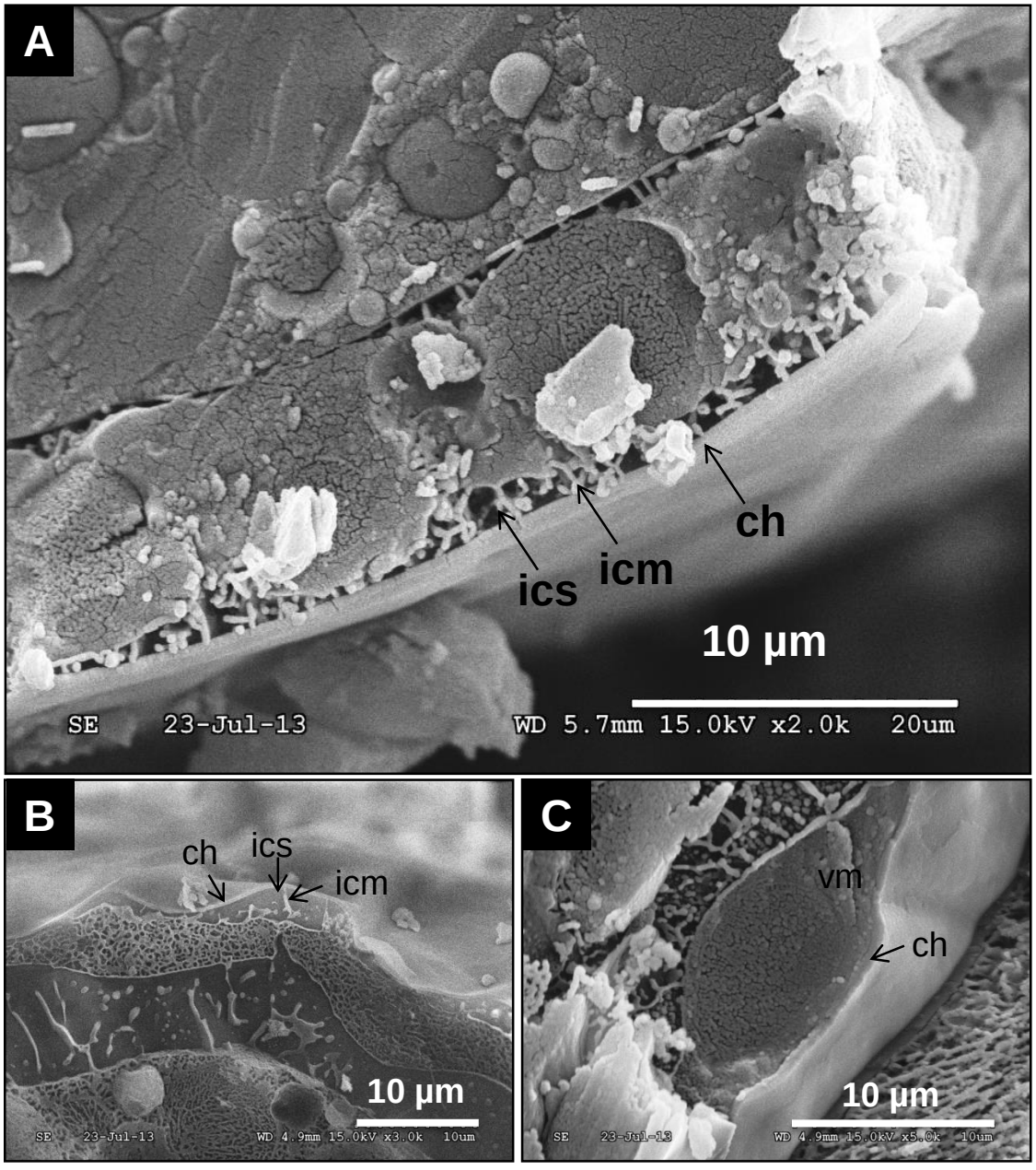
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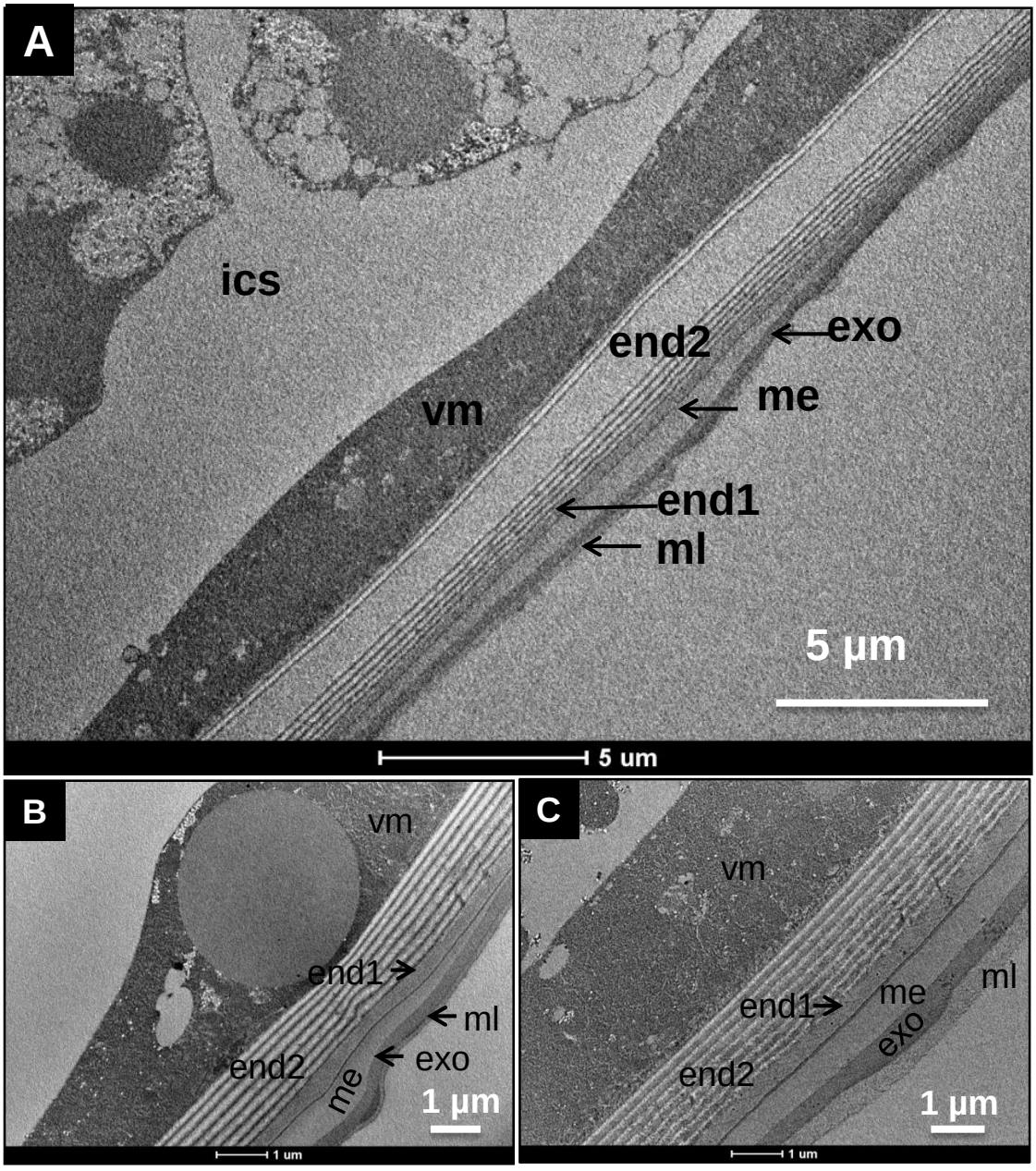
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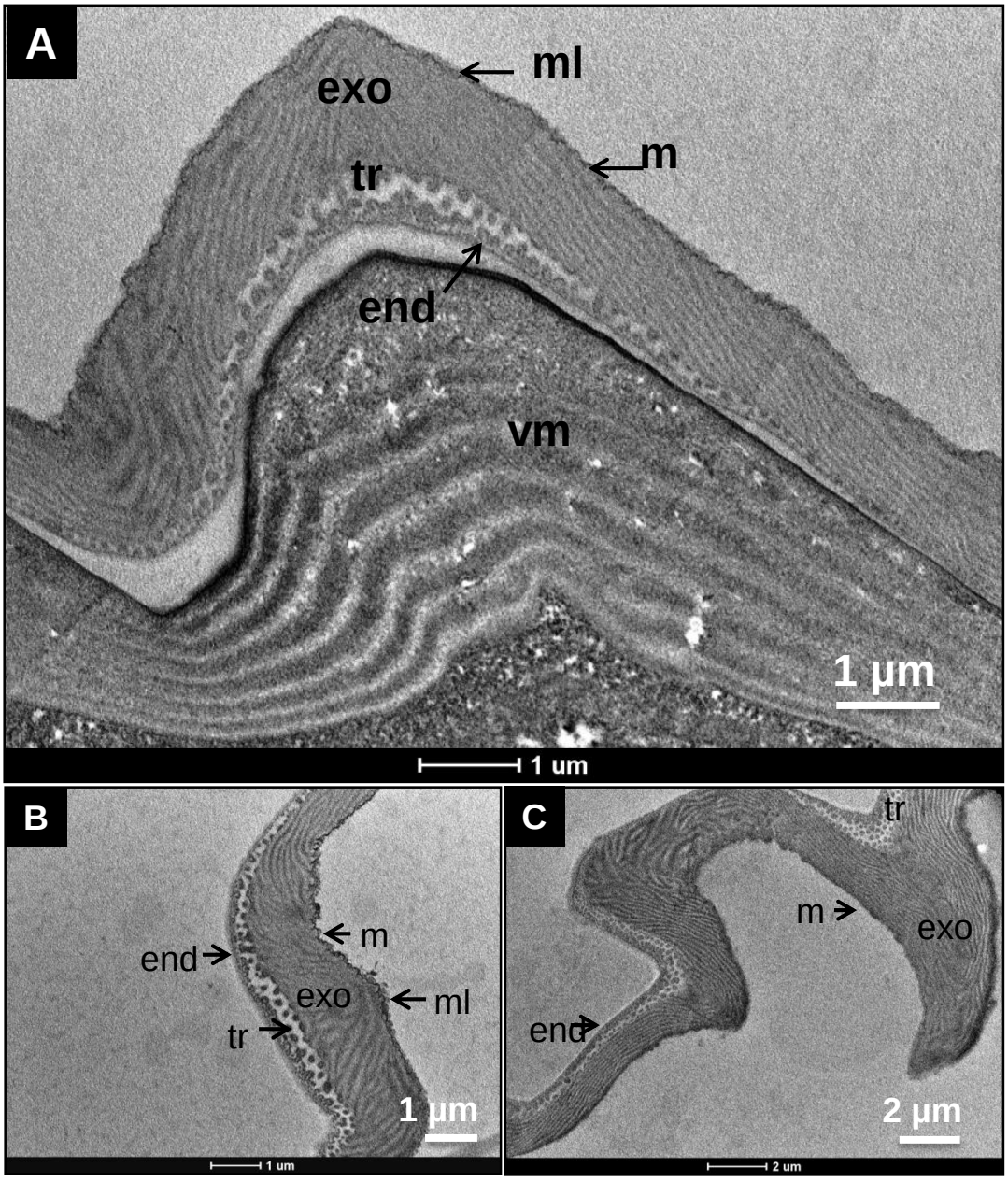
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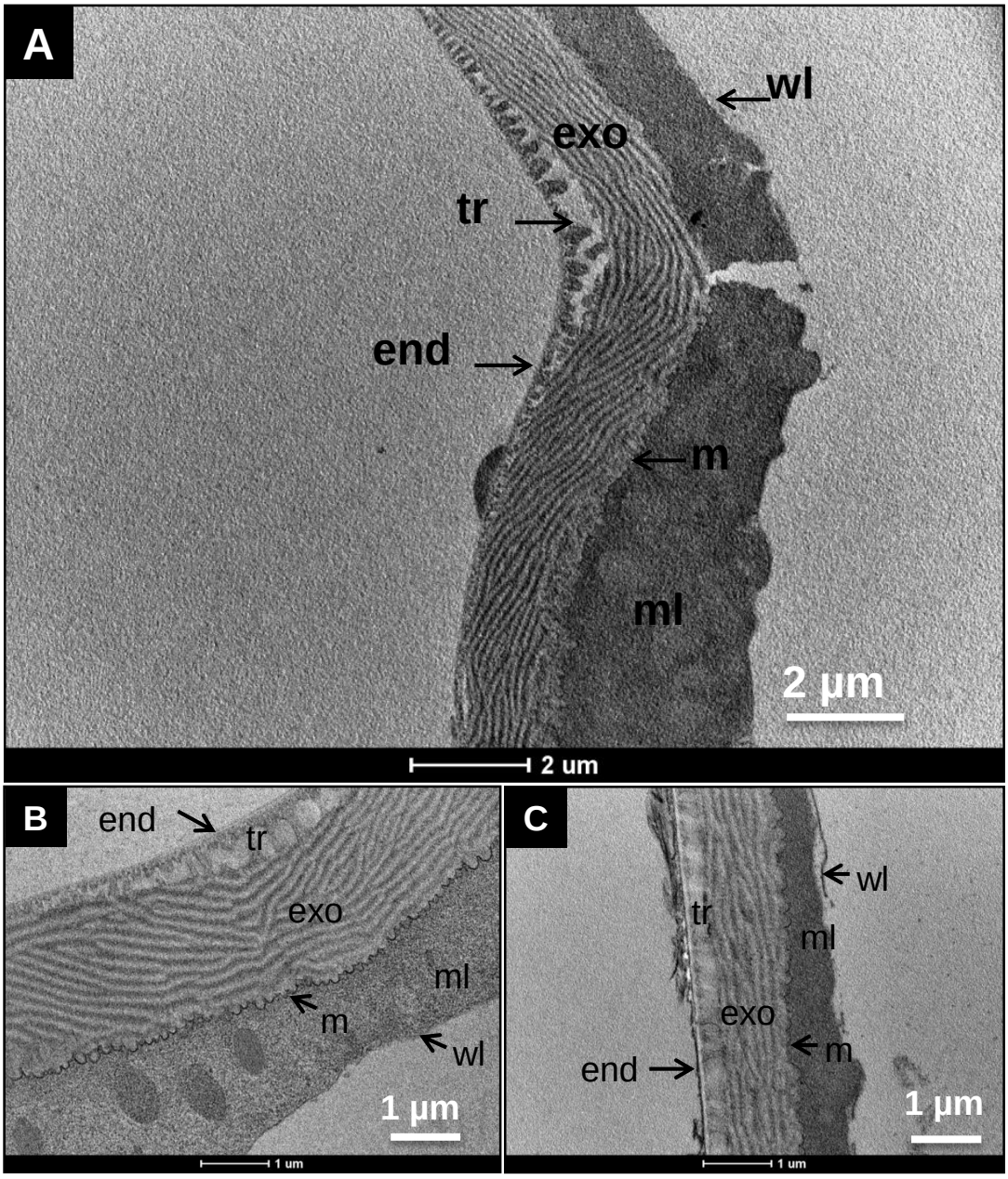
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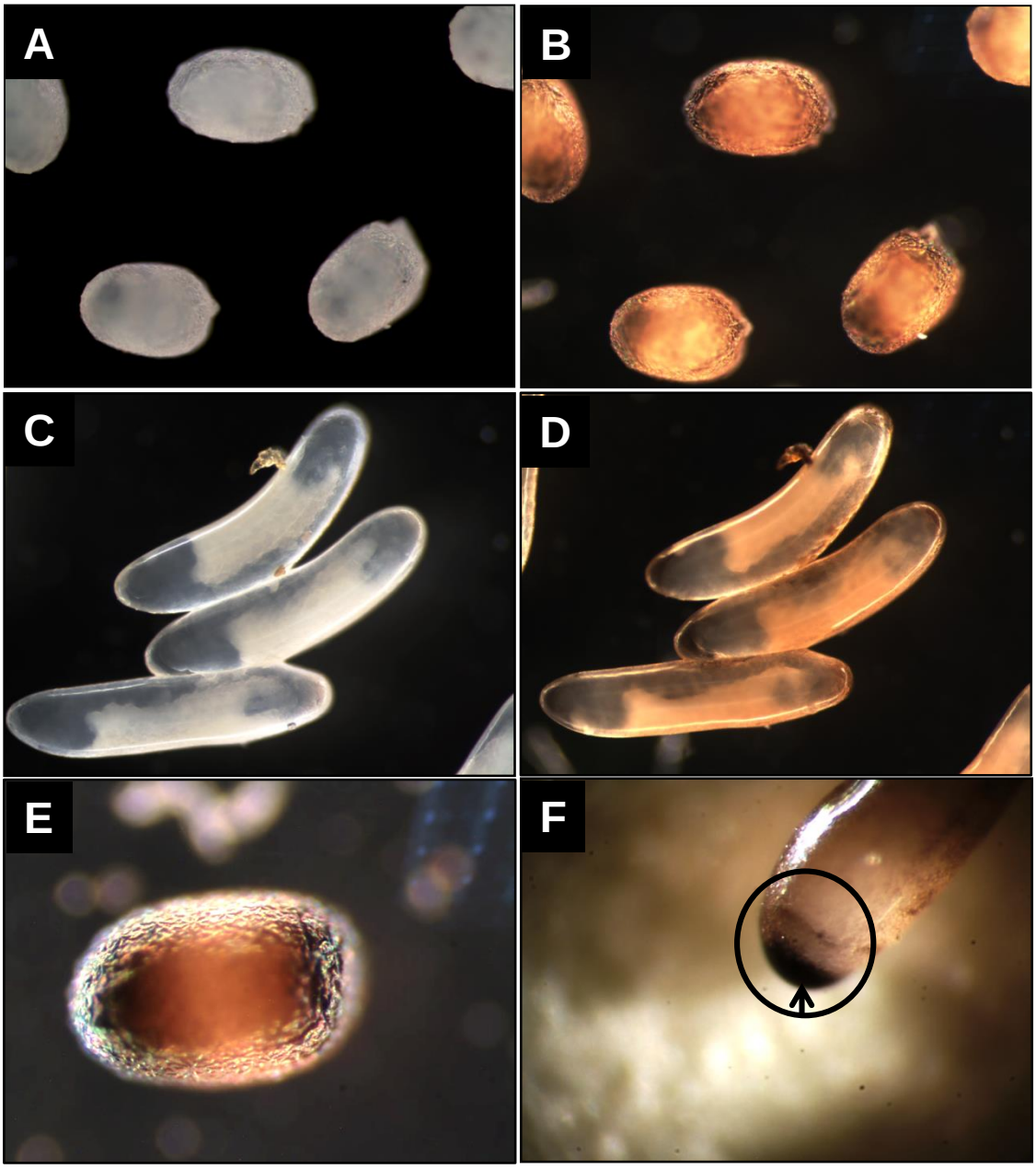
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CHAPTER V

EFFICACY OF PROPYLENE OXIDE AGAINST EGGS OF SEVEN POSTHARVEST INSECT PESTS AT LOW PRESSURE

(To be submitted to Journal of Economic Entomology)

Abstract

Because of the regulated phaseout of methyl bromide, California-based dried fruit and nut industries have transitioned to using sulfuryl fluoride (SF) where rapid disinfestations of insect pests are required. However, the ovicidal efficacy of SF is species specific and eggs of several key California pests are not adequately controlled by dose requirements listed on the current SF label for rapid disinfestation. The present work was conducted in the context of overcoming ovicidal deficiencies of SF in the California walnut industry that fumigates high value in-shell walnuts at low pressure (in vacuum). Efficacy of propylene oxide (PPO) against eggs of seven postharvest insect pests, namely, *Carpophilus hemipterus* (L.), *Tribolium castaneum* (Herbst), *Plodia interpunctella* (Hübner), *Ephestia elutell*a (Hübner), *Lasioderma serricorne* (F.), *Cydia pomonella* (L.), and *Amyelois transitella* (Walker) at 100 mmHg and 25°C was evaluated. LD_{50} mortalities for these species were 261.8, 79.9, 46.9, 42.0, 42.3, 23.6, and 22.1 mg h/liter, respectively. LD_{99} mortalities were 674.4, 170.4, 87.4, 75.9, 65.6, 67.2, and 49.3 mg h/liter, respectively. Generally, PPO was more toxic to lepidopteran eggs than to coleopteran eggs of the species tested. These results represent an initial step in formulating a blend of SF-PPO to meet disinfestation requirements of the California-based walnut industry for low pressure fumigations.

Keywords: fumigation, vacuum, quarantine, pre-shipment, toxicity.

California accounts for nearly all the United States' walnut production and in 2011 this was valued at $1.34 billion (NASS 2013). Most of this production is destined for foreign markets. For example, the annual export of fresh (in-shell) and dried (shelled) walnuts in 2011 was $1.04 billion (USDA ERS 2013). However, postharvest insect pests pose a serious risk to this lucrative California walnut industry. Field pests that are of postharvest concern include *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), and *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). Pests of concern during storage are *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae), and *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) (Johnson et al. 2009, Burks and Johnson 2012). As far as pest status is concerned, the California walnut industry ranks *A. transitella* and *C. pomonella* higher than the other five aforementioned species because they are quarantine pests which pose greater risk to the export of in-shell walnuts. Because *A. transitella* and *C. pomonella* are manually removed during shelling, they do not represent a major problem in shelled walnuts. Shelled walnuts are rarely infested by *C. hemipterus*. Postharvest fumigation is the only effective measure that can be used to disinfest walnuts of any field and storage pests that could be on them or are well hidden in the "meat" and are protected by the shell. Eggs of these pests are usually found on the surface whereas the postembryonic stages may inhabit deeper in the nut. As previously mentioned *A. transitella* and *C. pomonella* are the major pests of concern in freshly harvested in-shell walnuts that have to be rapidly disinfested and shipped to foreign markets or stored. Until recently, the walnut industry relied on only methyl bromide (MeBr or CH_3Br) for

disinfestation of freshly harvested walnuts to kill any pests that might be present at the time of harvest. Rapid disinfestation practiced by the industry is 4 h under vacuum or 24 h under normal atmospheric pressure (NAP). After the phaseout of MeBr, sulfuryl fluoride (SF or SF_2O_2) was adapted as a MeBr alternative for rapid disinfestations to guarantee quarantine security under time sensitive scenarios for disinfestation of freshly harvested walnuts for export or storage. Interestingly, vacuum fumigation is only used for export scenarios and there are approximately only 3 walnut processers that are capable of vacuum fumigation in the Central Valley of California. Currently, SF is the only MeBr alternative that is registered in the United States for vacuum fumigation (Walse et al. 2009).

Although highly effective against postembryonic stages of many stored-product pests (Bell and Savvidou 1999, Zettler and Arthur 2000, Baltaci et al. 2009), at ≤ 15°C, SF vacuum or NAP applications at the dose requirements listed on the current SF label (200 or 1500 mg h/liter, respectively) cannot achieve complete mortality of eggs of several walnut pests such as *A. transitella*, *C. pomonella*, *P. interpunctella*, *T. castaneum*, and *C. hemipterus* (S. W., unpublished data). In order to overcome the ovicidal deficiencies of SF, the current recommendations are using repeat applications of this fumigant in order to target newly hatched larvae or to extend the exposure period beyond egg incubation time (Johnson et al. 2012). However, SF fumigations in the United States are restricted by both dose requirements listed on the label and maximum residue limits (MRLs) on treated commodities (Johnson et al. 2012). The dose requirement listed on the current SF label for vacuum fumigation is 200 mg h/liter and the MRLs for inorganic fluoride and sulfuryl fluoride in walnuts are 10 and 3 ppm, respectively. Given the rapid

field disinfestation requirements associated with the marketing of in-shell walnuts, constraints imposed by the SF label dose, and MRL thresholds, it is urgent that ways to overcome the ovicidal deficiencies of SF, that take into consideration these three factors, be sought to facilitate its continued use for rapid postharvest disinfestations. Ovicidal limitations of SF, which can be circumvented by blending it with a potent ovicide during fumigation is a driving factor for the identification of a highly effective ovicide for this purpose. Propylene oxide (PPO) is a logical choice to blend with SF because it is already a proposed fumigant for quarantine and pre-shipment treatment based on the fact that it is a highly effective ovicide (Isikber et al. 2006). Studies on combining PPO with SF during fumigation have shown promising results (Muhareb et al. 2009).

PPO is a colorless liquid fumigant under normal environmental conditions, has a boiling point of 34.4°C, and a noticeable ether like odor (Griffith 1999). It has a very low environmental risk compared to MeBr because it does not deplete the ozone layer and degrades into nontoxic propylene glycol in soil and in the human stomach (Griffith 1999, Navarro et al. 2004). PPO is a FDA approved sterilant to kill bacteria, mold, and yeast contamination on processed spices, cocoa, and processed nutmeats except peanuts (Griffith 1999). It is a favored treatment method for pasteurizing almonds (Almond Board of California 2008). The major disadvantage of PPO as a fumigant is its flammability, which ranges from 2-36% by volume in air (Griffith 1999). This drawback can be overcome by fumigating under vacuum or in combination with CO_2 (Navarro et al. 2004). Studies on efficacy of PPO against *P. interpunctella* and *T. castaneum* at 100 mmHg have shown that PPO is a potent ovicide (Isikber et al. 2004a, 2004b; Navarro et al. 2004; Isikber et al. 2006). Interestingly, these studies have shown that concentrations

of PPO required to kill postembryonic stages of stored-product insects are much higher than those required to kill eggs, which is the opposite of SF that has ovicidal deficiencies but is highly effective against postembryonic stages.

Obtaining dose response relationships of PPO for pests that infest walnuts in California is a critical initial step in formulating a SF-PPO blend to meet the disinfestation requirements of the walnut industry for chamber fumigations under rapid disinfestation scenarios, i.e. ≤ 4 h under vacuum or ≈24 h under NAP. The purpose of this study was to determine concentration of PPO to be used in the SF-PPO blend for vacuum fumigation. Therefore, the first objective was to determine the dose response relationships of PPO at 100 mmHg for eggs of seven California walnut pests, namely, *C. hemipterus*, *T. castaneum*, *L. serricorne*, *P. interpunctella*, *E. elutella*, *C. pomonella*, and *A. transitella.* The second objective was to establish whether doses of PPO that were highly effective against eggs were also effective against postembryonic stages of the respective species.

Materials and Methods

Insects. Eggs of seven insect pest species, namely, *C. hemipterus*, *T. castaneum*, *L. serricorne*, *P. interpunctella*, *E. elutella*, *C. pomonella*, and *A. transitella*, were used for the experiment. All eggs required for fumigation were obtained from an insectary at the United States Department of Agriculture-Agricultural Research Service (USDA-ARS), San Joaquin Valley Agricultural Sciences Center (SJVASC), Parlier, CA. Voucher specimens of *C. hemipterus*, *T. castaneum*, *L. serricorne*, *P. interpunctella*, *E. elutella*, *A. transitella*, and *C. pomonella* adults which laid eggs used in this study were preserved in 95% ethyl alcohol and deposited at K. C. Emerson Entomology Museum at Oklahoma

State University under lot numbers 138, 139, 140, 141, 142, 144, and 143, respectively. Rearing conditions for all species were 27 ± 0.01°C (SE), 60 ± 0.24% RH (SE), and 16:8 (L:D) h. *Carpophilus hemipterus* were reared on ripe banana on top of sand substrate in 946-ml glass jars; *T. castaneum* on oats diet in 946-ml glass jars; *L. serricorne* on rice bran diet in 946-ml glass jars; *P. interpunctella*, *E. elutella*, and *A. transitella* on red flaky wheat bran diet in 3.8-liter glass jars; and *C. pomonella* on lima bean agar diet in 30-ml cups (USDA 2007). *Carpophilus hemipterus* culture was originally obtained in 1978 from Italian Swiss Colony Winery in Fresno county, CA; *T. castaneum* and *L. serricorne* in 1967 from an unknown source; *P. interpunctella* from a walnut packing house, Stanislaus Co., Modesto, CA; *E. elutella* in 1969 from USDA Tobacco Investigations Laboratory, Richmond, VA; *C. pomonella* in 1984 from Madera county, CA; and *A. transitella* in 1966 from University of California, Berkeley, CA (USDA 2012).

Obtaining Freshly Laid Eggs. *Carpophilus hemipterus*. Freshly laid eggs were obtained by placing 75 to 100 adults in a 237-ml glass jar. Glass slides (25 x 75 x 1 mm) were prepared as substrate for egg laying. A thin smear of lima bean agar diet was spread down the center (the 75 mm midline) of each slide into which two wax paper strips (25 x 75 mm) folded in half were placed on each side with the crease folds towards the center, i.e. covering the whole slide surface. Two slides were then bound together using a rubber band (#16) in such a way that wax papers were sandwiched between the glass slides and the edges of the slides were aligned. Four prepared slide units were placed in a 237-ml glass jar along with egg-laying females. The glass jar was covered with a moist 70-mm filter paper, a wire screen (U.S. Standard #40), a final moist filter paper covering, and all

were secured using a metal ring. Jars were placed in a holding room as described above for 12-18 h. Freshly laid 0- to 18-h-old eggs were collected and used for fumigation.

Tribolium castaneum and Lasioderma serricorne. One hundred to 200 *T. castaneum* adults were collected from the stock culture on a cardboard strip and transferred into a 946-ml glass jar containing wheat flour as a substrate. For *L. serricorne*, 100-200 adults were aspirated and placed in a 946-ml glass jar with wheat flour as a substrate. For both species, jars containing adult insects were covered with a wire screen (U.S. Standard #40) sandwiched between two pieces of 90-mm filter paper and secured using a metal ring. The jars containing adults were placed in the holding room with conditions as described above to lay eggs. After 3 d, eggs were harvested by sifting contents of jars containing the egg-laying insects through U.S. Standard #20 (0.84-mm openings) and #60 (0.25-mm openings) pair of sieves (Seedburo Equipment Company, Des Plaines, IL) for *T. castaneum*, and through U.S. Standard #25 (0.71-mm openings) and #70 (0.21-mm openings) pair sieves for *L. serricorn*e. Eggs that were 0 to 3 d old were used for fumigation.

Plodia interpunctella and *Ephestia elutella.* For each species, 100-200 adults were aspirated into a 1.9-liter glass jar that was covered with a wire screen (U.S. Standard #40) and 90-mm filter paper, and was secured with a metal ring. The jar was then inverted on top of a large paper clip spacer, which sat on a 10-cm Petri dish lined with a filter paper. The egg layers were placed in a holding room with conditions as described above for 3 d. After 3 d, 0- to 3-d-old eggs were collected and used for fumigation.

Cydia pomonella. Sixty adult *C. pomonella* were aspirated from 30-ml rearing cups containing lima bean agar diet and were transferred into a 2.1-liter stainless steel

can. The inner surface of the can was lined with velour poster board (Hygloss products Inc., Wallington, NJ). Both ends of the can were closed with wax papers held in place with plastic snap cap lids. The can was placed in a holding room with conditions as described above for 2 d. Eggs that were 0 to 2 d old were collected and used for fumigation.

Amyelois transitella. One hundred and fifty adult *A. transitella* were aspirated and transferred into a 2.3-liter stainless steel can and the can opening was covered with white paper towel that was secured to the container with the help of two rubber bands (#18). The setup was then placed on its side on an incubator shelf with the paper facing night light (4-watt bulb) in a holding room for 3 d. Eggs that were 0 to 3 d old were collected and used for fumigation.

Experimental Setup. *Eggs.* All insect eggs were counted under a Stereomicroscope (Zeiss SV 6, Carl Zeiss Microscopy, Thornwood, NY). Eggs of *C. hemipterus* were attached to glass slide units where they were counted. Five to six slides containing 200-300 eggs were placed inside a 237-ml glass jar and covered with a U.S. Standard #40 wire screen and a metal ring. Two hundred to 300 eggs of *T. castaneum*, *P. interpunctella*, or *L. serricorne* were placed on top of black velour paper that lined the inside of a 35-mm Petri dish (Falcon, Oxnard, CA). Because egg cases of these species usually get stuck on neonates and get carried around, black velour paper was used to contain empty egg cases within the experimental arenas in order to facilitate mortality assessment. Sheets of paper containing eggs *C. pomonella* or *A. transitella* (wax paper or paper towel) were cut into pieces containing 200-300 eggs each. Each piece of paper was then placed on top of an inverted Fisherbrand® polystyrene weighing boat (Fisher

Scientific, Pittsburg, PA). The 35-mm Petri dishes (that were used to set up *T. castaneum*, *P. interpunctella*, and *L. serricorne*) or the polystyrene weighing boats containing eggs were each placed at the center of a 10-cm Petri dish that contained red flaky bran diet on its periphery to trap neonates. In *E. elutella*, neonates are cannibalistic; therefore, to mitigate cannibalism, 200-300 eggs were spread on sticky tape that was attached to the bottom of a 10-cm Petri dish to trap neonates. For all insect species, each setup which had at least 200-300 eggs was placed on an aluminum tray inside each fumigation chamber.

Postembryonic stages. For *C. pomonella*, diapausing larvae were the only postembryonic stage tested. Larvae were handpicked from 30-ml cups with lima bean agar diet used for rearing *C. pomonella*, placed in 946-ml glass jars, and covered with a U.S. Standard #40 wire screen and a metal ring. For each of the other six species the larval, pupal, and adult postembryonic stages were tested. These postembryonic stages were obtained directly from the respective cultures of these species. Larvae and pupae of *T. castaneum* and *L. serricorne* were obtained by sieving with a U.S. Standard #30 (0.59-mm openings) sieve and placed on a 10-cm Petri dish. Larvae and pupae of *C. hemipterus*, *P. interpunctella*, *E. elutella*, or *A. transitella* were picked from the rearing jar using a blunt forceps, placed in a 237-ml glass jar, covered with a U.S. Standard #40 wire screen and secured using a metal ring. Adults of each of the seven species were aspirated into 237-ml glass jars. In all tests involving postembryonic stages, each setup had at least 50 individuals. Each 946-ml jar, Petri dish, or 237-ml jar containing individuals of the postembryonic stage to be tested was then placed on the aluminum tray of each chamber for fumigation.

Fumigation Chambers. Chambers used for fumigation were modified 28.3-liter Labconco® vacuum desiccators (Labconco® #5530000) (referred to as chambers hereafter). These were part of a multi-chamber manifold system that can support up to twelve 28.3-liter fiberglass chambers (Tebbets 2009). The entire system was housed inside a stainless steel room (17 x 17 x 17 m) with temperature and relative humidity controls (SJVASC fumigation facility; Tebbets 2009). The chambers are modified and contain three valves: a) connection to a manifold which functions in exhaust and/or vacuum, b) stopcock - to or from the side port to accommodate the gas syringe, and c) a toggle valve or septa (Tebbets 2009). For this experiment, the toggle valve (Swagelok®) on the bulkhead fitting (Swagelok®) was replaced with a 9.5-mm blue rubber septum (#6514 Altech Associates Inc, Deerfield, IL) for needle injection of PPO. Inside each chamber, just below the septum, an inverted 946-ml glass jar was placed. The top of the jar had a 10-cm glass Petri dish lined with a filter paper to receive liquid PPO injected into the chamber. Setups for eggs or postembryonic stages, described above, were placed on an aluminum tray which was part of each chamber. Stopcocks and door gaskets were sealed using high vacuum grease (Dow Corning®) and the doors closed and locked using door latches.

Propylene Oxide. Pure liquid propylene oxide (≥ 99.5%; #82320 Aldrich, Sigma-Aldrich Co. St. Louis, MO) was used. Propylene oxide was drawn from a 50-ml conical flask with a glass stopper under a certified fume hood using a 0.1-ml gas syringe (Hamilton, Foxboro/Analabs, North Haven, CT) or 1-, 2-, or 5- ml gas syringes (Precision syringe, Dynatech Precision Sampling, Baton Rouge, LA) befitting the applied dosages. The syringes Ire equipped with a small bore 5-cm long hypothermic needle.

Fumigation. *Four-Hour Egg Exposure*. Lethal concentrations (LCs) required to kill 50% (LC_{50}), 95% (LC_{95}), and 99% (LC_{99}) of *C. hemipterus* eggs were determined using PPO concentrations of 48, 56, 64, 80, 96, 128, and 160 mg/liter. Thereafter, LD_{50}, LD_{95}, and LD_{99} values were calculated. Prior to injecting liquid PPO into the chambers, a pressure of 20 mmHg was created. This ensured space for PPO volatilization and yet maintained the 100 mmHg pressure in the entire chamber. Applied dosages of PPO were pre-calculated and then injected into the chambers using a syringe, through the rubber septum. Liquid PPO was injected onto a filter paper on an open 10-cm glass Petri dish that sat on top of an inverted glass jar. The liquid PPO volatilized within 2-5 min. After the PPO volatilized, pressure inside chamber was adjusted to 100 mmHg by permitting air in through the stopcock. This marked the start of the 4-h fumigation exposure period. The concentration of PPO inside each chamber was determined at the end of the experiment, after pressure was normalized by letting air in through the stopcock valve. Gas samples were taken using a 100-ml gas syringe (Becton, Dickinson and Co., Franklin Lakes, NJ) by withdrawing 40 ml of gas through the stopcock. Because the experiment was conducted at reduced pressure, sampling could not be done at the beginning of the experiment. Concentrations of PPO in the chambers were quantitatively measured using a gas chromatograph (GC) (Model 3800, Varian Inc., Walnut Creek, CA).

Two-Hour Egg Exposure. Eggs of *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, and *A. transitella* were subjected to 2-h fumigations using an experimental procedure similar to the 4-h fumigations for *C. hemipterus*. Concentrations of PPO used to determine LC_{50}, LC_{95}, and LC_{99} for these six species were different and based on preliminary range-finder experiments (Table 1).

The concentrations of PPO in chambers were measured at the end of 2- and 4-h exposure periods and concentration x time (CT) (mg h/liter) products were calculated using the method of Bond (1984) (Table 2). All tests were conducted at 25 ± 1°C. In both the 2- and 4-h fumigations, for eggs of each species, there were three replications over time for each PPO concentration, i.e., three temporal replications. For each species, each PPO concentration replicate had two control treatment sets: one comprised of insects at 100 mmHg and the other at normal atmospheric pressure (NAP), i.e. each of these controls was replicated three times.

Two-Hour Postembryonic Stages Exposure. To determine the effects of PPO exposure on post-embryonic stages of *T. castaneum, P. interpunctella, E. elutella, L. serricorne, C. pomonella*, and *A. transitella*, the highest concentrations of PPO that were used in establishing their dose response were used (Table 1), namely, 96, 64, 48, 40, 32, and 32 mg/l, respectively. Mortality of postembryonic stages of *C. hemipterus*, *P. interpunctella, E. elutella, L. serricorne, C. pomonella*, and *A. transitella* caused by the highest concentration of PPO used in establishing the dose response of *T. castaneum* (96 mg/l) was also assessed. These tests were conducted at 100 mmHg for 2 h at 25°C. Fumigation and gas sampling procedures were as described above. For postembryonic stages of each species, there were three temporal replications of each PPO concentration. In all cases, there was only one replicate of each of the two control treatment sets.

*Fumigant Analysis***.** Concentrations of PPO were measured using a Varian gas chromatograph (GC), (Model 3800, Varian Inc., Walnut Creek, CA), equipped with a 1-cc gas sampling loop and a flame ionization detector (FID). Conditions for the GC were as follows: 2 mm internal diameter (ID) x 2 m teflon column packed with 10% OV-

101on Gas-Chrom Q (100/120 mesh). During the operation, gas flow rates were: helium carrier gas at 20 ml/min, hydrogen (FID) at 30 ml/min, air (FID) at 250 ml/min, oven temperature at 100°C, FID detector at 250°C, and gas sampling loop injection temperature at 110°C. With these conditions, the retention time of PPO was 1.5 min.

Post-Fumigation and Mortality Assessment. Immediately following gas sampling, chambers were aerated for 20-30 min. Egg setups were then transferred to a chamber maintained at 27 ± 1°C and 65 ± 5% RH and incubated. Jars containing *C. hemipterus* eggs were covered with a moist cloth towel to maintain >85% RH because eggs of this species are susceptible to dehydration. Egg mortality assessments were conducted after egg hatch. Mortality assessments for *C. hemipterus, T. castaneum, P. interpunctella, E. elutella, L. serricorne, C. pomonella,* and *A. transitella* eggs were conducted 3, 8, 5, 7, 10, 7, and 7 d, respectively, after fumigation. Incubation periods for eggs of these species had been pre-determined from preliminary experiments and were ≈1-2, 6-7, 3-5, 5-7, 8-10, 5-7, and 5-7 d, respectively. Mortality assessment of larvae and pupae due to PPO began with metamorphosis into next life stage and continued until all larvae pupated or pupae emerged as adults or were quantified as dead. Mortality of adults was assessed after 24 h.

Data Analysis. The experimental design for assessing the response of eggs to PPO was a randomized complete block design. In all cases, there were three temporal replications for each PPO concentration. Data for vacuum treated controls were used for the data analysis. Response of eggs of each of the seven species tested to PPO was subjected to probit analysis using PoloPlus (Leora Software, Petaluma, CA) to determine the LD_{50}, LD_{95}, and LD_{99} values and their 95% confidence intervals (CIs). Differences in

toxicity were considered significant when 95% CIs did not overlap. A ratio test to compare LDs was also conducted (Robertson et al. 2007). LD_{50}, LD_{95}, and LD_{99} values of *C. hemipterus, T. castaneum, L. serricorne, P. interpunctella, E. elutella,* or *C. pomonella* eggs were compared with those of *A. transitella* eggs which were consistently the lowest. Probit analyses were used because probit transformation of the proportion kill resulted in the best fit to the data (Leora Software 2005). The experimental design for assessing the mortality of postembryonic stages caused by PPO was a randomized complete block design with three temporal replications. All post-embryonic stages did not survive the PPO concentrations tested hence data analyses were not conducted. Because there was only one replicate of each of the two control treatment sets, standard error values for mortalities of postembryonic stages are not reported.

Results

Gas Concentrations in Fumigation Chambers. The concentration of PPO in each chamber was measured at the end of the 2- or 4-h fumigation period (Table 2). Concentration x time (CT) products were then calculated (Table 2).

Egg Response to Propylene Oxide. Propylene oxide at 100 mmHg was toxic to eggs of all the seven species tested, but there were differences in response among species (Fig. 1; Table 3). LD_{99} values for eggs of the seven species ranged from 49.3-674.4 mg h/liter (Table 3). Generally, eggs of lepidopterans tested were less tolerant to PPO compared to eggs of coleopterans; *L. serricorne* was the exception. LD_{99} values for *A. transitella, C. pomonella, E. elutella,* and *P. interpunctella* eggs were 49.3, 67.2, 75.9, and 87.4 mg h/liter, respectively; these values were lower than those for *T. castaneum* and *C. hemipterus* eggs which were 170.4 and 674.4 mg h/liter, respectively (Table 3).

Interestingly, the LD_{99} for eggs of *L. serricorne*, a coleopteran species, was relatively low (65.6 mg h/liter) and not different from LD_{99} values of the lepidopteran species (Fig. 1; Table 3). Based on LD_{50} or LD_{99} comparisons, eggs of *C. hemipterus* required a significantly higher PPO dose to kill than eggs of other species. *T. castaneum* eggs require a significantly higher dose to kill than *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, and *A. transitella* eggs (Table 3). LD_{99} values for eggs of *P. interpunctella, L. serricorne, E. elutella,* and *C. pomonella* were not different but were significantly higher than that of *A. transitella* eggs (Table 3). Based on LD_{50} values from the 2- and 4-h fumigations conducted at 100 mmHg, the tolerance to PPO for the species tested in decreasing order was *C. hemipterus* > *T. castaneum* > *P. interpunctella* ≥ *E. elutella* ≥ *L. serricorne* > *C. pomonella* ≥ *A. transitella* (Fig. 1; Table 3). LD_{50}, LD_{95}, and LD_{99} values of *C. hemipterus*, *T. castaneum*, *L. serricorne*, *P. interpunctella*, *E. elutella,* and *C. pomonella* eggs were compared with those of *A. transitella* to determine the degree of their tolerance to PPO relative to eggs of *A. transitella* (Table 4). Data on mortality of control eggs at 100 mmHg and at NAP for all life stages are reported (Table 5).

Post embryonic stages. The highest concentration of PPO that was used in establishing the dose response of *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, or *A. transitella* eggs (Table 1) resulted in 100% mortality of all postembryonic stages in the respective species. Additionally, the highest concentration of PPO that was used in establishing the dose response of *T. castaneum* eggs (96 mg/l) resulted in 100% mortality of postembryonic stages of *C. hemipterus*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella,* and *A. transitella*. For all the seven species, 100 mmHg alone did not have significant effects on postembryonic life stages, except in

adults. Data on mortality of postembryonic stages at 100 mmHg and at NAP for all life stages are reported (Table 5). *C. hemipterus* adults did not survive low pressure conditions of 100 mmHg for 2 h. The highest postembryonic mortality for all species in the NAP control treatment was 4.2%, in *T. castaneum* pupae.

Discussion

The present study provides data on responses of eggs of seven postharvest insect pest species, namely, *C. hemipterus*, *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, and *A. transitella* to PPO at 100 mmHg. Results show that PPO at 100 mmHg is an effective ovicide. The LD_{50} values for *C. hemipterus*, *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, and *A. transitella* were 261.8, 79.9, 46.9, 42.0, 42.3, 23.6, and 22.1 mg h/liter, respectively. The LD_{99} values for these species were 674.4, 170.4, 87.4, 75.9, 65.6, 67.2, and 49.3 mg h/liter, respectively.

As previously mentioned, two pests of most concern to the California walnut industry are *A. transitella* and *C. pomonella*. PPO LC_{99} values for eggs of these pests are 24.7 and 33.6 mg/liter, respectively. According to this data, probit 9 (P9) PPO concentrations for *A. transitella* and *C. pomonella* were 43.9 and 71.3 mg/liter, respectively. P9 concentrations for *A. transitella* and *C. pomonella* for 2-h fumigation at 25°C in this study are much lower than FDA recommended concentrations for the pasteurization of almonds (500 mg/l). All almonds must be pasteurized before marketing and fumigating with PPO is a favored treatment method, especially for the mitigation of *Salmonella* populations (Almond board of California 2008). The FDA recommended pasteurization treatment for almonds is 500 mg/l of PPO at 630 mmHg for 4 h at a product temperature of 47-51°C (Almond Board of California 2008). The relatively much

lower PPO LC_{99} and P9 concentrations for *A. transitella* and *C. pomonella* applied for only 2 h may mean that residue levels left in the walnuts are probably low and favor PPO as a potential fumigant to blend with SF to achieve high egg mortality of these two pests.

It is important that the concentration of PPO that will be blended with SF also be highly effective against eggs of *T. castaneum*, *P. interpunctella*, *E. elutella*, and *L. serricorne*, in case they happen to be infesting in-shellwalnuts under vacuum fumigation. *C. hemipterus* is excluded because it is extremely rare for it to be found infesting in-shellwalnuts destined for vacuum fumigation (on rare occasions could infest shelled walnuts). PPO LC_{99} values for *T. castaneum*, *P. interpunctella*, *E. elutella*, and *L. serricorne* eggs were 85.2, 43.7, 38.0, and 32.8 mg/liter, respectively. Among these four species, LC_{99} values for *T. castaneum* were the highest and were 3.5- and 2.5-fold higher than those of *A. transitella and C. pomonella*, respectively. It can be assumed that fumigating walnuts with eggs of these six species using 85 mg/liter of PPO at 100 mmHg for 2 h would kill all their eggs. Similar to what was stated for *A. transitella* and *C. pomonella*, the LC_{99} concentration for *T. castaneum* (85 mg/liter) applied over a 2-h period is much lower than 500 mg/liter applied for 4 h for almond sterilization. Therefore, studies are suggested determine absorbance and the levels of PPO residues (PPO and propylene chlorohydrin) left in walnuts after fumigation using 85 mg/liter of PPO at 100 mmHg for 2 h in order to establish the feasibility of blending PPO at this concentration with SF to achieve high mortality of all stages of these six pest species.

SF dose response studies at 100 mmHg have shown that 8 mg/liter of this fumigant will result in ≥ 99% mortality of all postembryonic stages of *C. hemipterus*, *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, and *A. transitella*

(S.W., unpublished data). According to the SF and PPO information provided above, it seems logical that a PPO:SF blend composed of 85 mg/liter of PPO and 8 mg/liter of SF be tested at 100 mmHg for 2 h for its efficacy against all stages of *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, and *A. transitella*.

Based on LD_{50} values, the tolerance to PPO of the seven species tested in decreasing order was determined as *C. hemipterus* > *T. castaneum* > *P. interpunctella* ≥ *E. elutella* ≥ *L. serricorne* > *C. pomonella* ≥ *A. transitella.* Data from SF dose response studies conducted at 100 mmHg on eggs of *C. hemipterus, T. castaneum*, *P. interpunctella*, *C. pomonella*, and *A. transitella* show a similar order of tolerance for these species as data in the present study (S. W., unpublished data). For example, at 15.6°C, SF LD_{50} values for these species were 3319.8, 589.7, 330.7, 268.4, and 127.1 mg h/liter, respectively (S. W., unpublished data). Based on these data, it seems eggs of the coleopteran species tested require higher doses of PPO and SF to exert a similar level of mortality compared to eggs of lepidopteran species, with the exception of *L. serricorne* eggs treated with SF (Su and Scheffrahn 1990) and PPO.

The reason for higher PPO LD_{50} and LD_{99} values for *C. hemipterus* and *T. castaneum* eggs compared to eggs of the other five species may partly be the presence of a much smaller number of respiratory openings or lack of respiratory openings on their egg surfaces compared to the other five species. Gautam et al. (2014) showed that *C. hemipterus* has only two aeropyles located at the tip of the anterior end of each egg and no micropyle. In *T. castaneum* eggs, there are neither aeropyles nor micropyles present (unpublished data). On the other hand, *A. transitella*, *L. serricorne*, and *P. interpunctella* eggs have many aeropyles and several micropyles, and each *E. elutella* and *C. pomonella*

egg has many aeropyles and a single micropyle (Fehrenbach et al. 1987, Gautam et al. 2014, unpublished data). Although confirmatory measurements of fumigant diffusion into eggs are needed to show that the presence and number of respiratory openings on the eggs surface influence fumigant entry and efficacy, it is plausible to suggest that the order of tolerance of eggs of the seven species tested to PPO may be partly influenced by the surface morphology of eggs and that chorionic respiratory structures on them differentially affect fumigant penetration and/or uptake (Gautam et al. 2014).

In conclusion, this study has provided data on responses of eggs of seven stored-product species, namely, *C. hemipterus*, *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, and *A. transitella* to PPO at 100 mmHg. Based on these results, it is suggested that studies be conducted using a PPO:SF blend composed of 85 mg/liter of PPO and 8 mg/liter of SF at 100 mmHg for 2 h to determine its efficacy against all stages of *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, and *A. transitella*. Studies should also be conducted to determine levels of PPO residues left in walnuts after fumigation using 85 mg/liter of PPO at 100 mmHg for 2 h in order to establish whether they are within acceptable limits. The SF-PPO blend this study has recommended for testing represents an initial but important step in finding an alternative to MeBr for for rapid field disinfestation scenarios.

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Table 1. Concentrations of PPO (mg/liter) tested against eggs of *T. castaneum, P. interpunctella, E. elutella, L. serricorne, C. pomonella,* and *A. transitella.*

T. castaneum	*P. interpunctella*	*E. elutella*	*L. serricorne*	*C. pomonella*	*A. transitella*
32	16	12	16	8	8
40	20	16	18	10	10
48	24	20	20	12	12
56	32	24	24	16	16
64	40	32	28	20	20
80	48	40	32	24	24
96	64	48	40	32	32

Table 2. Concentrations (mean ± SE) and concentration x time "CT" products of PPO at 2- and 4-h exposure.

Tested concentrations of	Concentrations of PPO mg/liter		Calculated CT products (mg h/liter)	
PPO	2-h exposure*	4-h exposure*	2-h exposure	4-h exposure
160	N/A	149.5 ± 0.49	N/A	619.1 ± 0.8
128	N/A	124.7 ± 0.58	N/A	505.4 ± 0.9
96	89.6 ± 1.09	91.4 ± 1.50	185.2 ± 1.5	374.8 ± 2.4
80	75.8 ± 1.04	75.4 ± 1.16	155.7 ± 1.2	310.7 ± 1.9
64	59.7 ± 0.39	63.4 ± 0.45	123.9 ± 0.6	254.7 ± 0.7
56	53.8 ± 1.25	57.0 ± 0.29	110.0 ± 1.6	230.3 ± 1.5
48	47.1 ± 0.91	45.8 ± 0.67	96.3 ± 3.1	187.6 ± 1.1
40	37.1 ± 1.01	N/A	76.9 ± 1.5	N/A
32	29.2 ± 0.49	N/A	61.3 ± 0.9	N/A
28	25.8 ± 0.70	N/A	53.9 ± 1.3	N/A
24	21.8 ± 0.59	N/A	45.9 ± 1.0	N/A
20	19.1 ± 0.73	N/A	39.7 ± 1.8	N/A
18	17.1 ± 0.69	N/A	35.7 ± 1.7	N/A
16	15.1 ± 0.41	N/A	31.8 ± 1.3	N/A
12	12.0 ± 0.42	N/A	24.9 ± 1.5	N/A
10	10.5 ± 0.72	N/A	21.9 ± 1.9	N/A
8	7.7 ± 0.33	N/A	16.9 ± 1.0	N/A

*N/A, these PPO concentrations were not tested for specified time exposures.

Table 3. Probit analyses of mortality for eggs of *C. hemipterus, T. castaneum, P. interpunctella, E. elutella, L. serricorne, C. pomonella,* and *A. transitella* after exposure to propylene oxide at 25°C and 100 mmHg. Lethal doses are concentration x time (CT) (mg h/liter) products.

	n	Slope ± SE	LD_{50}**	LD_{90} (95% CI)	LD_{95}	LD_{99}**	χ^2
*C. hemipterus**	5532	5.7 ± 0.17	261.8a (242.4-279.9)	440.9 (403.9-497.1)	511.2 (459.4-594.5)	674.4a (582.1-835.6)	54.4
T. castaneum	5315	7.1 ± 0.26	79.9b (75.3-83.5)	121.3 (115.9-127.3)	136.4 (129.5-145.2)	170.4b (158.5-186.9)	17.6
P. interpunctella	6686	8.5 ± 0.25	46.9c (43.9-49.6)	66.1 (62.2-72.5)	72.9 (67.5-81.3)	87.4c (78.8-102.2)	72.6
E. elutella	13381	8.7 ± 0.24	42.0c (38.6-44.6)	58.2 (54.6-63.6)	63.8 (59.3-74.0)	75.9c (68.7-94.9)	125.0
L. serricorne	6771	12.1 ± 0.34	42.3c (40.6-43.9)	53.9 (51.7-56.9)	57.7 (54.9-61.8)	65.6c (61.4-71.8)	68.1
C. pomonella	5277	5.1 ± 0.15	23.6d (22.2-25.0)	42.1 (39.2-45.9)	49.5 (45.4-55.2)	67.2c (59.7-78.6)	24.9
A. transitella	6027	6.7 ± 0.22	22.1d (20.4-23.2)	33.4 (32.7-36.4)	38.9 (36.7-41.8)	49.3d (44.2-54.6)	23.8

*For *C. hemipterus,* exposure time was 4 h. For all other species, exposure time was 2 h.

**LD values within a column followed by different letters are significantly different based on overlap of 95% CI.

Table 4. Comparisons of lethal doses (concentration x time) required to kill 50, 95, or 99% of *A. transitella* eggs* to those required to kill eggs of *C. pomonella, L. serricorne, E. elutella, P. interpunctella, T. castaneum,* and *C. hemipterus*.

Lethal dose ratios	*C. pomonella*	*L. serricorne*	*E. elutella* (95% CI)	*P. interpunctella*	*T. castaneum*	*C. hemipterus***
LD_{50}	1.07	1.92	1.90	2.13	3.61	11.87
	(1.04-1.11)	(1.87-1.97)	(1.85-1.96)	(2.06-2.19)	(3.48-3.75)	(11.47-12.27)
LD_{95}	1.27	1.48	1.64	1.77	3.50	13.12
	(1.21-1.34)	(1.43-1.53)	(1.58-1.70)	(1.68-1.87)	(3.36-3.65)	(12.51-13.77)
LD_{99}	1.62	1.33	1.54	1.56	3.46	13.68
	(1.43-1.83)	(1.27-1.40)	(1.47-1.62)	(1.41-1.71)	(3.26-3.67)	(12.79-14.64)

*Because *A. transitella* eggs had consistently lower LD values indicating it was the most susceptible, lethal doses of other species were compared to those of *A. transitella*.

**For *C. hemipterus*, exposure time was 4 h. For all other species, exposure time was 2 h.

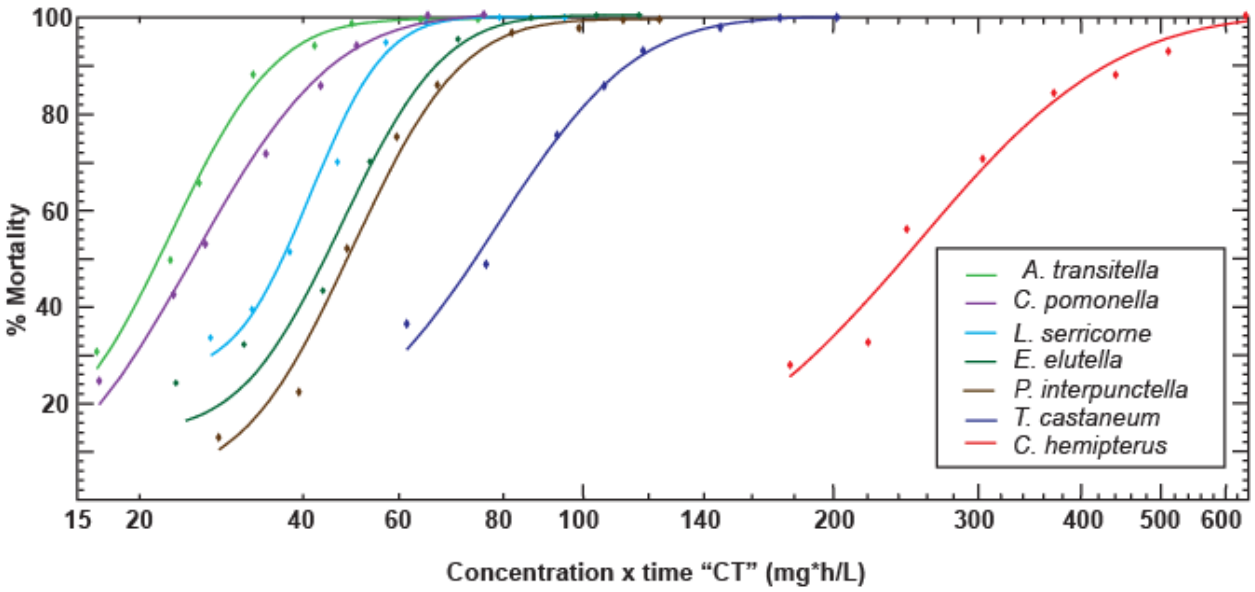
Table 5. Mortality of all four life stages of *C. hemipterus, T. castaneum, P. interpunctella, E. elutella, L. serricorne, C. pomonella,* and *A. transitella* in the untreated control treatments, i.e., insects treated at 100 mmHg and normal atmospheric pressure (NAP).

Species	Life Stages	% mortality	
		100 mmHg	NAP
C. hemipterus	Eggs	9.5 ± 4.9	6.6 ± 3.1
	Larvae	0.0	0.0
	Pupae	13.2	N/A*
	Adults	100.0	0.0
T. castaneum	Eggs	12.9 ± 3.6	7.5 ± 1.6
	Larvae	0.4	0.2
	Pupae	6.9	4.2
	Adults	0.2	0.4
P. interpunctella	Eggs	5.2 ± 1.8	1.3 ± 0.3
	Larvae	0.0	0.0
	Pupae	3.2	1.6
	Adults	41.7	1.3
E. elutella	Eggs	6.5 ± 2.6	2.7 ± 1.9
	Larvae	3.9	1.9
	Pupae	8.1	0.0
	Adults	91.1	0.0
L. serricorne	Eggs	3.4 ± 0.6	0.9 ± 0.3
	Larvae	0.0	0.0
	Pupae	2.4	0.3
	Adults	5.8	0.7
C. pomonella	Eggs	15.0 ± 1.2	13.7 ± 1.0
	Diapausing larvae	N/A	1.8
A. transitella	Eggs	10.6 ± 2.2	12.4 ± 2.1
	Larvae	5.3	0.0
	Pupae	2.9	2.3
	Adults	70.1	2.8

*N/A, the specified life stage was not tested.

Figure caption

Fig. 1. Dose response relationships of propylene oxide at 100 mmHg and 25°C for *C. hemipterus*, *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C. pomonella*, and *A. transitella* eggs. Fumigations for eggs of *C. hemipterus* lasted 4 h whereas it was 2 h for the other six species.


100
80
60
40
20
% Mortality
15
20
40
60
80
100
140
200
300
400
500
600
Concentration x time "CT" (mg*h/L)
A. transitella
C. pomonella
L. sericorne
E. elutella
P. interpunctella
T. castaneum
C. hemipterus

CHAPTER VI

Efficacy of Propylene Oxide in Combination with Carbon Dioxide Against Eggs of Six Postharvest Insect Pests

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Abstract. Sulfuryl fluoride (SF) is increasingly used as a methyl bromide alternative for postharvest disinfestation of dried fruits and nuts in the United States. However, SF is a species-specific ovicide and is not effective against eggs of several key California pests. The present work was conducted in the context of circumventing ovicidal deficiency of SF in order to facilitate its continued use in postharvest disinfestations by California's walnut industry. The efficacy of propylene oxide (PPO) in combination with carbon dioxide (CO_2) against eggs of six postharvest insect pests, namely, *Carpophilus hemipterus* (L.), *Tribolium castaneum* (Herbst), *Lasioderma serricorne* (F.), *Amyelois transitella* (Walker) *Ephestia elutell*a (Hübner), and *Plodia interpunctella* (Hübner) at 25°C and normal atmospheric pressure was evaluated. Efficacy of PPO alone against eggs of *C. hemipterus*, *T. castaneum*, *A. transitella*, and *P. interpunctella* under the same conditions were also evaluated. Results show that PPO alone or in combination with CO_2 was toxic to eggs of all the insect species tested. Generally, coleopteran eggs required higher concentrations of PPO to kill compared to lepidopteran eggs. LD_{50} mortalities of PPO in combination with CO_2 for *C. hemipterus*, *T. castaneum*, *L. serricorne*, *A. transitella*, *E. elutella*, and *P. interpunctella* eggs were 193.4, 174.8, 71.2, 51.2, 49.7, and 47.4 mg h/liter, respectively. LD_{99} mortalities were 414.8, 348.6, 113.1, 133.1, 104.7, and 95.3 mg h/liter, respectively. These results represent a critical and initial step in formulating a blend of SF-PPO to meet disinfestation requirements of the California walnut industry.

Keywords: fumigation, quarantine, stored product, methyl bromide, sulfuryl fluoride

The annual production of dried fruits and nuts in the central valley of California is > 2,000,000 metric tons, valued at ≈$18 billion. This accounts for nearly all of the dried fruits and nuts produced in the United States (USDA ERS 2013). California's walnut production in 2011 was valued at $1.34 billion, and $1.1 billion of this comprised exports. In-shell and shelled walnuts exported during this period were valued at $466 and 646 million, respectively (NASS 2013, USDA ERS 2013). However, several postharvest insect pests pose a serious threat to California's walnut production. Field pests that are of key concern in in-shellwalnuts are *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) and *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Johnson et al. 2009, Burks and Johnson 2012). These two pests are quarantine pests in several countries that import in-shellwalnuts. Pests of concern during storage are *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae), and *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) (Johnson et al. 2009, Burks and Johnson 2012).

Postharvest chamber fumigation using methyl bromide (MeBr) is the most effective tool available to the walnut industry for rapid disinfestation immediately after harvest to ensure quarantine and commodity protection (Schneider et al. 2003, Johnson et al. 2012). Quarantine treatments of walnuts are required by importing countries to prevent introduction of exotic pests. Therefore, postharvest chamber fumigation of in-shell walnuts is designed to eliminate all life stages of *A. transitella* and *C. pomonella*. For >50 yr., MeBr was the fumigant of choice for postharvest disinfestation of walnuts to ensure pest free security. After the phaseout of MeBr, the walnut industry transitioned to using SF for rapid disinfestation of in-shell walnuts. A drawback to using SF at the

recommended label rate, i.e., 1,500 mg h/liter at NAP or 200 mg h/liter at vacuum, is that it is not effective against eggs of several pests of walnuts (S.W., unpublished data, UNEP 2011). Therefore ways to circumvent ovicidal deficiencies of SF in postharvest fumigations need to be found. One possible solution is blending SF with another fumigant that is highly effective against eggs.

Propylene oxide is a logical choice for blending with SF because it is a FDA-approved sterilant for pasteurizing almonds, and has been shown to be an effective ovicide (Isikber et al. 2004a 2004b, Navarro et al. 2004, Isikber et al. 2006). In fact, a 1:1 combination of PPO and SF has been shown to be effective against all life stages of *T. castaneum* (Muhareb et al. 2009). Other fumigation studies using PPO at vacuum have shown it to be effective against stored-product insect eggs (unpublished data). PPO has a very low environmental risk as ozone depleter and is degraded into nontoxic propylene glycol in soil and in the human stomach (Griffith 1999, Navarro et al. 2004). However, its major disadvantage is flammability, which can be reduced by combining it with low pressure or CO_2 (8:92 – PPO:CO2) (Navarro et al. 2004). This clearly indicates it is a potent ovicide that could be mixed with SF to produce a blend that is highly effective against all stages of *C. pomonella*, *A. transitella*, *T. castaneum*, *P. interpunctella*, *E. elutella*, and *L. serricorne* at 100 mmHg for 2 h.

As previously mentioned, chamber fumigation is the most effective tool available for rapid field disinfestations of walnut pests. Disinfestations that are not associated with export of in shell walnuts are carried out at NAP for ≈24 h. After regulated phaseout of MeBr, California's walnut industry has transitioned to using SF at NAP or at vacuum for rapid postharvest disinfestations of walnuts. However, the recommended label rate for SF

fumigation at NAP (1,500 mg h/liter), is not effective against eggs of several insect pests that infest walnuts (UNEP 2011 and references therein). Identifying concentrations of PPO that are effective against eggs of stored-product insect pests at NAP would facilitate the formulation of a SF-PPO blend that could be used effectively for rapid disinfestation scenarios against all stages of insect pests that infest walnuts. Therefore, the first objective was to establish the response of *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae), *T. castaneum, L. serricorne, E. elutella, A. transitella, P. interpunctella*, *C. pomonella*, and *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae) to PPO in combination with CO_2 (8% PPO and 92% CO_2) at 25°C and NAP. The second objective was to determine the response of *C. hemipterus, T. castaneum, A. transitella,* and *P. interpunctella* eggs to PPO alone at 25°C and NAP.

Materials and Methods

Insects. Eggs of eight insect pest species, namely, *C. hemipterus, T. castaneum, L. serricorne, P. interpunctella, E. elutella, A. transitella, C. pomonella,* and *E. ceratoniae* were utilized. All eggs required for the experiment were obtained from an insectary at the United States Department of Agriculture-Agricultural Research Service (USDA-ARS), San Joaquin Valley Agricultural Sciences Center (SJVASC), Parlier, CA. Voucher specimens of *C. hemipterus, T. castaneum, L. serricorne, P. interpunctella, E. elutella, A. transitella, C. pomonella,* and *E. ceratoniae* adults which laid eggs used in this study were preserved in 95% ethyl alcohol and deposited at K. C. Emerson Entomology Museum at Oklahoma State University under lot numbers 138, 139, 140, 141, 142, 143, 144, and 145, respectively. Rearing conditions for all species were 27 ± 0.01°C (SE), 60 ± 0.24% RH (SE), and 16:8 (L:D) h. *C. hemipterus* were reared on ripe

banana laid on top of sand substrate in 946-ml glass jars; *T. castaneum* on oats diet in 946-ml glass jars; *L. serricorne* on rice bran diet in 946-ml jars; *P. interpunctella*, *E. elutella*, *A. transitella*, and *E. ceratoniae* on red flaky wheat bran diet in 3.8-liter glass jars; *C. pomonella* on lima bean agar diet in 30-ml cups (USDA 2007). *C. hemipterus* culture was originally obtained in 1978 from Italian Swiss Colony Winery in Fresno county, CA; *T. castaneum*, *E. ceratoniae*, and *L. serricorne* in 1967 from an unknown source; *P. interpunctella* from a walnut packing house, Stanislaus Co., Modesto, CA; *E. elutella* in 1969 from USDA Tobacco Investigations Laboratory, Richmond, VA; *A. transitella* in 1966 from University of California, Berkeley, CA; *C. pomonella* in 1984 from Madera county, CA (USDA 2012).

Obtaining Freshly Laid Eggs. *Carpophilus hemipterus*. Freshly laid eggs were obtained by placing 75 to 100 adults in a 237-ml glass jar. Glass slides (25 x 75 x 1 mm) were prepared as substrate for egg laying. A thin smear of lima bean agar diet was spread down the center (the 75 mm midline) of each slide into which two wax paper strips (25 x 75 mm) folded in half were placed on each side with the crease folds towards the center, i.e. covering the whole slide surface. Two slides were then bound together using a rubber band (#16) in such a way that wax papers were sandwiched between the glass slides and the edges of the slides were aligned. Four prepared slide units were placed in a 237-ml glass jar along with egg-laying females. The glass jar was covered with a moist 70-mm filter paper, a wire screen (U.S. Standard #40), a final moist filter paper covering, and all were secured using a metal ring. Jars were placed in a holding room as described above for 12-18 h. Freshly laid 0- to 18-h-old eggs were collected and used for fumigation.

Tribolium castaneum and Lasioderma serricorne. One hundred to 200 *T. castaneum* adults were collected from the stock culture on a cardboard strip and transferred into a 946-ml glass jar containing wheat flour as a substrate. For *L. serricorne*, 100-200 adults were aspirated and placed in a 946-ml glass jar with wheat flour as a substrate. For both species, jars containing adult insects were covered with a wire screen (U.S. Standard #40) sandwiched between two pieces of 90-mm filter paper and secured using a metal ring. The jars containing adults were placed in the holding room with conditions 27 ± 0.01°C (SE), 60 ± 0.24% RH (SE), and 16:8 (L:D) h to lay eggs. After 3 d, eggs were harvested by sifting contents of jars containing the egg-laying insects through U.S. Standard #20 and # 60 (0.84- and 0.25-mm openings, respectively) pair of sieves (Seedburo Equipment Company, Des Plaines, IL) for *T. castaneum*, and through U.S. Standard #25 and #70 (0.71- and 0.21-mm openings, respectively) pair sieves for *L. serricorn*e. Eggs that were 0 to 3 d old were used for fumigation.

Plodia interpunctella and *Ephestia elutella.* For each species, 100-200 adults were aspirated into a 1.9-liter glass jar that was covered with a wire screen (U.S. Standard #40) and 90-mm filter paper, and was secured with a metal ring. The jar was then inverted on top of a large paper clip spacer, which sat on a 10-cm Petri dish lined with a filter paper. The jars were placed in a holding room with conditions as described above for 3 d. After 3 d, 0- to 3-d-old eggs were collected and used for fumigation.

Cydia pomonella. Sixty adult *C. pomonella* were aspirated from 30-ml rearing cups containing lima bean agar diet and were transferred into a 2.1-liter stainless steel can. The inner surface of the can was lined with velour poster board (Hygloss products Inc., Wallington, NJ). Both ends of the can were closed with wax papers held in place

with plastic snap cap lids. The can was placed in a holding room with conditions as described above for 2 d. Eggs that were 0 to 2 d old were collected and used for fumigation.

Amyelois transitella. One hundred and fifty adult *A. transitella* were aspirated and transferred into a 2.3-liter stainless steel can and the can opening was covered with white paper towel that was secured to the container with the help of two rubber bands (#18). The setup was then placed on its side on an incubator shelf with the paper facing night light (4-watt bulb) in a holding room for 3 d. Eggs that were 0 to 3 d old were collected and used for fumigation.

Ectomyelois ceratoniae. Eggs were collected by hanging 5 x 10-cm wax paper strips in a 3.8-liter rearing jar that contained newly emerged adults. Wax paper strips each folded lengthwise to make crease folds were hung on a wire frame that was above wheat bran diet in the jar. The jar was covered with a 110-mm filter paper, a wire screen (U.S. Standard #40), and a final a filter paper. The filter papers and wire screen were secured using a metal lid with a 70-mm hole at the center. The jar was placed in a holding room with conditions as described above for 3 d. After 3 d, 0- to 3-d-old eggs were collected and used for fumigation.

Experimental Setup. *Eggs.* All insect eggs were counted under a Stereomicroscope (Zeiss SV 6, Carl Zeiss Microscopy, Thornwood, NY). Eggs of *C. hemipterus* were attached to glass slide units where they were counted. Five to six slides containing 200-300 eggs were placed inside a 237-ml glass jar and covered with a U.S. Standard #40 wire screen and a metal ring. Two hundred to 300 eggs of *T. castaneum*, *P. interpunctella,* or *L. serricorne* were placed on top of black velour paper that lined the

inside of a 35-mm Petri dish (Falcon, Oxnard, CA). Because egg cases of these species usually get stuck on neonates and get carried around, black velour paper was used to contain empty egg cases within the experimental arenas in order to facilitate mortality assessment. Paper towel containing *A. transitella* eggs and sheets of wax papers containing *C. pomonella* or *E. ceratoniae* eggs were cut into pieces that had 200-300 eggs each. Each piece of paper was then placed on top of an inverted Fisherbrand® polystyrene weighing boat (Fisher Scientific, Pittsburg, PA). The 35-mm Petri dishes (that were used to set up *T. castaneum*, *P. interpunctella*, and *L. serricorne*) or the polystyrene weighing boats containing eggs were each placed at the center of a 10-cm Petri dish that contained red flaky bran diet on its periphery to trap neonates. In *E. elutella*, neonates are cannibalistic; therefore, to mitigate cannibalism, 200-300 eggs were spread on sticky tape that was attached to the bottom of a 10-cm Petri dish to trap neonates. For all insect species, each setup which had at least 200-300 eggs was placed on an aluminum tray inside each fumigation chamber.

Fumigation Chambers. Chambers used for fumigation were modified 28.3-liter Labconco® vacuum desiccators (Labconco® # 5530000) (referred to as chambers hereafter). These were part of a multi-chamber manifold system that can support up to twelve 28.3- liter fiberglass chambers (Tebbets 2009). The entire system was housed inside a stainless steel room (17 x 17 x 17 m) with temperature and relative humidity controls (SJVASC fumigation facility; Tebbets 2009). The chambers are modified and contain three valves: a) connection to a manifold which functions in exhaust and/or vacuum, b) stopcock - to or from the side port to accommodate the gas syringe, and c) a toggle valve or septa (Tebbets 2009). For this experiment, the toggle valve (Swagelok®)

on the bulkhead fitting (Swagelok®) was replaced with a 9.5-mm blue rubber septum (#6514 Altech Associates Inc, Deerfield, IL) for needle injection of PPO. Inside each chamber, just below the septum, an inverted 946-ml glass jar was placed. The top of the jar had a 10-cm glass Petri dish lined with a filter paper to receive liquid PPO injected into the chamber. Setups for eggs, described above, were placed on an aluminum tray which was part of each chamber. Stopcocks and door gaskets were sealed using high vacuum grease (Dow Corning®) and the doors closed and locked using door latches.

The Fumigant. *Propylene Oxide.* Pure liquid propylene oxide (≥ 99.5%; #82320 Aldrich, Sigma-Aldrich Co. St. Louis, MO). Propylene oxide was drawn from a 50-ml conical flask with a glass stopper under a certified fume hood using a 0.1-ml gas syringe (Hamilton, Foxboro/Analabs, North Haven, CT) or 1-, 2-, or 5- ml gas syringes (Precision syringe, Dynatech Precision Sampling, Baton Rouge, LA) befitting the applied dosages. The syringes were equipped with a small bore 5-cm long hypothermic needle.

Carbon Dioxide. Carbon dioxide was drawn from a tank (13.4 x 45.7 cm) containing compressed CO_2 using 500-, 1000-, or 1,500-ml gas syringes. The required volume of CO_2 was obtained from the cylinder through a regulator which controlled flow of gas to a tube which was connected to a syringe of appropriate size.

Fumigation. *PPO: CO_2 (8:92).* Lethal concentrations (LCs) required to kill 50% (LC_{50}), 95% (LC_{95}), and 99% (LC_{99}) of *C. hemipterus*, *T. castaneum*, *L. serricorne*, *A. transitella*, *E. elutella*, and *P. interpunctella* were defined using PPO concentrations determined from preliminary range finder experiments (Table 1). Thereafter, LD_{50}, LD_{95}, and LD_{99} values were calculated. Responses of *C. pomonella* and *E. ceratoniae* eggs to PPO were determined using concentrations of 7.94 and 11.18 mg/liter. Preliminary range

finder experiments had determined these values as LC_{50} and LC_{90}, respectively, for *T. castaneum* eggs.

Prior to injecting liquid PPO into the chambers, a pressure of 250 mmHg was created. This ensured space for CO_2 and PPO volatilization while preventing the development of positive pressure in the entire chamber. Conditions to simulate 8:92 mixture of PPO:CO_2 were created by injecting a calculated volume of CO_2 equivalent that which would have been administered into each chamber in a scenario where the gas introduced was premixed PPO:CO_2 (8:92 mixture). Subsequently, pre-calculated volumes of liquid PPO were then injected into the chambers, through rubber septa, using syringes of different sizes, to attain desired concentrations. Liquid PPO was injected onto a filter paper on an open 10-cm glass Petri dish that sat on top of an inverted glass jar. The liquid PPO volatilized within 2-5 min. After the PPO volatilized, the pressure inside the chamber was adjusted to normal by permitting air in from the stopcock. This marked the start of the 24-h fumigation exposure period.

The concentrations of PPO inside each chamber were determined after the pressure was normalized at the start of each fumigation (0 h) and after 2 and 24 h. Gas samples were taken using a 100-ml gas syringe (Becton, Dickinson and Co., Franklin Lakes, NJ) by withdrawing 40 ml of gas through the stopcock. Concentrations of PPO in fumigation chambers were quantitatively monitored and analyzed using a gas chromatograph (GC) (Model 3800, Varian Inc., Walnut Creek, CA). The GC was equipped with a 1-cc gas sampling loop and a flame ionization detector (FID). Conditions for the GC were as follows: 2 mm internal diameter (ID) x 2 m teflon column packed with 10% OV-101on Gas-Chrom Q (100/120 mesh). During the operation, gas flow rates

were: helium carrier gas at 20 ml/min, hydrogen (FID) at 30 ml/min, air (FID) at 250 ml/min, oven temperature at 100°C, FID detector at 250°C, and gas sampling loop injection temperature at 110°C. With these conditions, the retention time of PPO was 1.5 min. Concentrations of CO_2 were monitored after 0, 2, and 24 h of fumigation. An oxygen/carbon dioxide analyzer (Model 902D, Quantek Instruments, Inc., Grafton, MA) was used to monitor CO_2 concentrations. Doses of PPO expressed as concentration x time (CT) product (mg h/liter) were calculated by the method of Bond (1984) (Table 2). All tests were conducted at 25°C.

PPO Alone. Eggs of *C. hemipterus, T. castaneum, A. transitella*, and *P. interpunctella* were subjected to 24-h PPO fumigations to determine their LC_{50}, LC_{95}, and LC_{99} values. Experimental procedures were similar to those described above except CO_2 was not added. Concentrations of PPO tested for each species were based on preliminary range finder experiments (Table 1). For *A. transitella* eggs, a PPO concentration of 8 mg/liter was also tested because preliminary results of PPO alone fumigation showed that complete mortality of *A. transitella* was not achieved with 6 mg/liter of PPO.

Post-Fumigation and Mortality Assessment. Immediately after the 24-h gas sampling, chambers were aerated for 20-30 min. Egg setups were then transferred to a chamber maintained at 27 ± 1°C and 65 ± 5% RH and incubated. Jars containing *C. hemipterus* eggs were covered with a moist cloth towel to maintain >85% RH because eggs of this species are susceptible to dehydration. Egg mortality assessments were conducted after egg hatch. Mortality assessments for *C. hemipterus, T. castaneum, P. interpunctella, E. elutella, L. serricorne, A. transitella, C. pomonella,* and *E. ceratoniae* eggs were conducted 3, 8, 5, 7, 10, 7, 7, and 7 d, respectively, after fumigation.

Incubation periods for eggs of these species had been pre-determined from preliminary experiments and were ≈1-2, 6-7, 3-5, 5-7, 8-10, 5-7, 5-7, and 5-7 d, respectively.

Data Analysis. The experimental design for assessing the response of eggs to PPO was a randomized complete block design. In all cases, there were three replications over time for each PPO concentration for each species tested, i.e., three temporal replications. Responses of eggs of the respective species tested to PPO:CO_2 or PPO alone were subjected to probit analysis using PoloPlus (Leora Software, Petaluma, CA) to determine the LD_{50}, LD_{95}, and LD_{99} values and their 95% confidence intervals (CIs). Differences in toxicity were considered significant when 95% CIs did not overlap. A ratio test to compare LDs was also conducted (Robertson et al. 2007). LD_{50}, LD_{95}, and LD_{99} values of *C. hemipterus*, *T. castaneum*, *L. serricorne*, *E. elutella*, and *A. transitella* eggs were compared with those of *P. interpunctella* eggs which were consistently the lowest. Probit analyses were used because probit transformation of the proportion kill resulted in the best fit to the data (Leora Software 2005). Because *C. pomonella* and *E. ceratoniae* eggs did not survive PPO concentrations of 7.94 and 11.18 mg/liter, data analyses were not conducted.

Results

Gas Concentrations in Fumigation Chambers. Concentrations of PPO in each chamber were measured after 0-, 2-, and 24-h exposures. PPO concentrations decreased over time, but 24-h concentrations of CO_2 were not different from starting (0 h) concentrations (Table 2). Concentration x time (CT) products were then calculated (Table 2).

Egg Response to Propylene Oxide in Combination with Carbon Dioxide.

Propylene oxide in combination with CO_2 was toxic to eggs of all the six species tested, but there were differences in responses among species (Fig. 1; Table 3). LD_{99} values for eggs of the six species ranged from 95.3-414.8 mg h/liter (Table 3). In general, lower doses of PPO were required to attain the same level of mortality for lepidopteran eggs compared to coleopteran eggs; *L. serricorne* was the exception. LD_{99} values for *P. interpunctella, E. elutella,* and *A. transitella* eggs were 95.3, 104.7, and 133.1 mg h/liter, respectively; these values were lower than those for *T. castaneum* and *C. hemipterus* eggs which were 348.6 and 414.8 mg h/liter, respectively (Table 3). Interestingly, the LD_{99} for eggs of *L. serricorne,* a coleopteran species, was relatively low (113.1 mg h/liter) and not different from LD_{99} values of the lepidopteran species (Fig. 1; Table 3). Based on LD_{50} or LD_{99} comparisons, eggs of *C. hemipterus* and *T. castaneum* required a significantly higher PPO dose to kill than eggs of other species (Fig. 1; Table 3). LD_{50} values of *L. serricorne* were higher than those for *A. transitella, E. elutella,* and *P. interpunctella*. But LD_{99} values of *L. serricorne* were not different than LD_{99} values for *E. elutella* and *A. transitella* (Table 3). Based on LD_{50} values from 24-h fumigations conducted at normal atmospheric pressure, the tolerance to PPO for the species tested in decreasing order was *C. hemipterus* ≥ *T. castaneum* > *L. serricorne* > *A. transitella* ≥ *E. elutella* ≥ *P. interpunctella* (Fig. 1; Table 3). LD_{50}, LD_{95}, and LD_{99} values of *C. hemipterus, T. castaneum, L. serricorne, A. transitella,* and *E. elutella* eggs were compared with those of *P. interpunctella* to determine the degree of their tolerance to PPO relative to eggs of *P. interpunctella* (Table 4). Propylene oxide concentrations of 7.94 and 11.18 mg/liter resulted in 100% mortality of *C. pomonella* and *E. ceratoniae* eggs.

Egg Response to Propylene Oxide Alone. Propylene oxide alone was toxic to eggs of *P. interpunctella, A. transitella, T. castaneum,* and *C. hemipterus,* but there were differences in responses among coleopteran and lepidopteran species (Table 5). LD_{99} values for eggs of the four species tested ranged from 90.0-477.9 mg h/liter (Table 5). Lower doses of PPO were required to attain the same level of mortality for *P. interpunctella* and *A. transitella* eggs compared to *T. castaneum* or *C. hemipterus* eggs. In fumigations involving PPO alone, LD_{99} values for *P. interpunctella*, *A. transitella*, *T. castaneum*, and *C. hemipterus* were 90.0, 166.7, 324.5, and 477.9 mg h/liter, respectively (Tables 5, and 6). The LD_{99} values for eggs of these species in fumigations involving a mixture of PPO and CO_2 (8:92) were 95.3, 133.1, 348.6, and 414.8 mg h/liter, respectively (Tables 3 and 6). LD_{50} and LD_{99} values for these species when fumigated with PPO alone or a mixture of PPO and CO_2 were not different based on overlap of CIs, except in the case of LD_{50} for *P. interpunctella* (Table 6).

Discussion

This study has shown that a combination of PPO and CO_2 (8:92), applied at NAP for 24 h, is effective against eggs of *C. hemipterus, T. castaneum, L. serricorne, A. transitella, E. elutella,* and *P. interpunctella*. PPO when applied alone at NAP for 24 h was also effective against eggs of *C. hemipterus, T. castaneum, A. transitella,* and *P. interpunctella*. Additionally, this study has demonstrated that concentrations of 7.94 and 11.18 mg/liter of PPO, in a PPO and CO_2 (8:92) mixture, applied at NAP for 24 h, are effective against *C. pomonella* and *E. ceratoniae* eggs. Among eggs of the stored-product species tested, eggs of *T. castaneum* were the most difficult to kill and required the highest PPO dose. On the other hand eggs of *P. interpunctella* were the least difficult to

kill. Propylene oxide LC_{99} concentrations for *T. castaneum* and *P. interpunctella* were 14.5 and 4.0 mg/liter, respectively. The P9 concentrations for these species were 23.8 and 6.6 mg/liter, respectively. These LC_{99} and P9 concentrations are much lower than the FDA recommended PPO concentration for pasteurization of almonds. For pasteurization of almonds, 500 mg/liter of PPO applied for a 4-h period, at a temperatures 47-51°C is recommended (Almond Board of California 2008).

It is significant that PPO LC_{99} and P9 concentrations that are effective against all the species tested are much lower than those recommended for the pasteurization of almonds because it implies that the PPO and CO_2 (8:92) mixture has potential to be used as a fumigant to blend with SF in order to achieve high egg mortality during rapid field disinfestations of walnuts at NAP. The PPO: SF blend to be developed should be effective against eggs of all field and stored-product pests. Although statistically similar, concentrations required to kill *C. hemipterus* were numerically higher than those required for *T. castaneum*. LC_{99} and P9 values for *C. hemipterus* and *T. castaneum* were 17.3 and 29.9 mg/liter and 14.5 and 23.8 mg/liter, respectively. Based on these data, investigating the effectiveness of 18 mg/liter of PPO, in a PPO and CO_2 (8:92) mixture, applied at NAP for 24 h, against eggs of *C. hemipterus, T. castaneum, L. serricorne, A. transitella, E. elutella, C. pomonella, E. ceratoniae,* and *P. interpunctella* is recommended. This would establish the feasibility of blending 18 mg/liter of PPO with SF for the control of all stages of the aforementioned eight postharvest pests during field disinfestations at NAP. Therefore, further studies on in-shell walnuts to determine PPO absorbance and the levels of its residues, namely, PPO and propylene chlorohydrins, based on fumigation using 18 mg/liter for 24 h at NAP are recommended. In addition, studies of PPO

absorbance and residues in shelled walnuts would be needed to formulate a blend of SF-PPO to be used for fumigating shelled walnuts in storage.

SF dose responses at NAP have shown that ≈8 mg/liter of this fumigant applied for 24 h will result in ≥ 99% mortality of all postembryonic stages of *C. hemipterus, T. castaneum, P. interpunctella, E. elutella, L. serricorne, C. pomonella,* and *A. transitella* (S.W., unpublished data, Su and Scheffrahn 1990, Leesch and Zettler 2000, Zettler and Arthur 2000, Baltaci et al. 2009). Therefore, it seems logical that a PPO:SF blend composed of 18 mg/liter of PPO, in a PPO and CO_2 (8:92) mixture, and 8 mg/liter of SF applied for 24 h at NAP needs to be tested for its efficacy against all stages of *C. hemipterus, T. castaneum, L. serricorne, A. transitella, E. elutella, C. pomonella, E. ceratoniae,* and *P. interpunctella.* Given that these eight species infest other dried fruits and nuts besides walnuts, the PPO efficacy data this study has generated could be used to explore ways to disinfest these commodities as well. For example, the date industry uses MeBr under CUE for field disinfestations of freshly harvested dates that need to be shipped to markets promptly. This is because dates have to be shipped to markets within 3 d of harvest in order to fetch a premium price and due to the fact that SF cannot kill eggs of *C. hemipterus* and *E. ceratoniae* at the label rate. Based on results from the current study, the concentration of PPO recommended for the SF-PPO blend at NAP can control eggs of *C. hemipterus* and *E. ceratonidae,* two key field pests of dates. Therefore, the recommended blend of SF-PPO at NAP is potentially a postharvest alternative to MeBr for rapid disinfestations of other dried fruits and nuts commodities as well.

Based on LD_{50} values obtained in the present study, eggs of coleopteran species with exception of *L. serricorne* required much higher doses of PPO for control than eggs

of lepidopteran species. The relative tolerance of eggs of coleopteran species to fumigants has also been demonstrated in PPO toxicity studies conducted at low pressure (Isikber et al. 2004b, Gautam et al., unpublished data) and SF studies at NAP and at low pressure (S. W., unpublished data, Su and Scheffrahn 1990). These differences in egg responses to fumigants may be explained partly by differences in respiratory structures facilitating fumigant uptake/penetration (Gautam et al. 2014). Although confirmatory measurements of fumigant diffusion into eggs via aeropyles and micropyles are needed to demonstrate a possible relationship, information on egg morphology of different stored-product insect species and data on efficacy of different fumigants for the control of these eggs suggests a possible link (Gautam et al. 2014). Eggs of *T. castaneum* do not have any aeropyles or micropyles (Gautam et al., unpublished data). *C. hemipterus* has 2 aeropyles and no micropyles and *E. elutella* has several aeropyles and a micropyle (Gautam et al. 2014). *L. serricorne*, *A. transitella*, *C. pomonella*, and *P. interpunctella* eggs have several aeropyles and micropyles (Fehrenbach et al. 1987, Gautam et al. 2014, Gautam et al. unpublished data).

These results also show that LD values for species tested using PPO alone or a PPO and CO_2 (8:92) mixture were not significantly different based on the overlap of CIs. This suggests that CO_2 did not have a synergistic effect. LC_{50} values for PPO alone against eggs of *C. hemipterus*, *T. castaneum*, *A. transitella*, and *P. interpunctella* were 1.09, 1.05, 0.8, and 1.15 times, respectively, greater compared with LC_{50} values for the PPO and CO_2 (8:92) mixture. Contrary results were shown by Navarro et al. (2004) who found that in 4-h fumigations at 30 ± 2°C using PPO alone, the LC_{50} value for *T. castaneum* eggs was 5.3 times greater than that for the PPO and CO_2 (8:92) mixture

hence indicating a synergistic effect of CO_2 in the mixture. Differences in exposure time and temperature during fumigations may explain this discrepancy in results.

This study has provided data on the responses of eggs of six stored-product species, namely, *C. hemipterus, T. castaneum, L. serricorne, A. transitella, E. elutella,* and *P. interpunctella* to the PPO and CO_2 (8:92) mixture. This study also demonstrated that *C. pomonella* and *E. ceratoniae* are can be controlled by ≈8 mg/liter of PPO. Based on these data, it is suggested that studies be conducted using a PPO:SF blend composed of 18 mg/liter of PPO, in a PPO and CO_2 (8:92) mixture, and 8 mg/liter of SF applied for 24 h at NAP to determine its efficacy against all stages of *C. hemipterus, T. castaneum, L. serricorne, A. transitella, E. elutella, C. pomonella, E. ceratoniae,* and *P. interpunctella*. Studies should also be conducted on in-shell and shelled walnuts to determine PPO absorbance and the levels of its residues, namely, PPO and propylene chlorohydrins, based on fumigation using 18 mg/liter for 24 h at NAP. The PPO:SF blend recommended for testing represents an initial but important step in finding an alternative to MeBr for rapid disinfestation of walnuts at NAP.

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Table 1. Concentrations (mg/liter) of propylene oxide tested against eggs of *C. hemipterus*, *T. castaneum*, *L. serricorne*, *P. interpunctella*, *E. elutella*, *A. transitella*, *C. pomonella*, and *E. ceratoniae.*

Coleoptera			Lepidoptera				
C. hemipterus	*T. castaneum*	*L. serricorne*	*P. interpunctella*	*E. elutella*	*A. transitella*	*C. pomonella*	*E. ceratoniae*
6.0	4.0	2.75	1.5	1.5	1.5	7.94	7.94
8.0	6.0	3.0	2.0	2.0	2.0	11.18	11.18
10.0	8.0	3.25	2.5	2.5	2.5	-	-
12.0	10.0	3.5	3.0	3.0	3.0	-	-
14.0	12.0	4.0	3.5	3.5	3.5	-	-
16.0	14.0	4.5	4.0	4.0	4.0	-	-
18.0	16.0	5.0	5.0	5.0	5.0	-	-
-	-	6.0	6.0	6.0	6.0	-	-

Table 2. Calculated concentrations (mean ± SE) of propylene oxide and carbon dioxide at 0- and 24-h exposures, and concentration x time (CT) products (mean ± SE) after 24-h exposure.

Concentrations (mg/liter) Tested Concentrations of PPO	Calculated CT (mg h/liter)					
	PPO		CO_2		24-h exposure	
	0 h	24 h	0 h	24 h	PPO	CO_2
1.5	1.62 ± 0.18	1.06 ± 0.13	13.8 ± 0.6	13.8 ± 0.6	32.1 ± 3.7	330.9 ± 14.4
2.0	2.10 ± 0.12	1.61 ± 0.17	19.8 ± 0.0	19.8 ± 0.0	44.5 ± 3.9	474.8 ± 0.0
2.5	2.45 ± 0.15	1.79 ± 0.11	23.4 ± 0.0	23.4 ± 0.0	51.0 ± 3.1	561.2 ± 0.0
2.75	2.55 ± 0.05	2.23 ± 0.13	22.1 ± 2.4	22.1 ± 2.4	57.4 ± 2.1	532.4 ± 57.5
3.0	2.95 ± 0.16	2.08 ± 0.10	27.6 ± 0.6	27.6 ± 0.6	60.3 ± 3.1	661.9 ± 14.4
3.25	3.45 ± 0.04	3.02 ± 0.16	26.9 ± 1.7	26.9 ± 1.7	74.8 ± 1.1	647.5 ± 43.2
3.5	3.47 ± 0.08	2.96 ± 0.45	30.6 ± 0.0	30.6 ± 0.0	77.6 ± 1.8	733.8 ± 0.0
4.0	4.44 ± 0.11	3.59 ± 0.23	29.4 ± 5.8	34.8 ± 0.6	95.8 ± 5.2	899.3 ± 58.9
4.5	4.51 ± 0.06	4.29 ± 0.28	37.8 ± 1.8	37.8 ± 1.8	103.3 ± 2.9	906.5 ± 43.2
5.5	5.28 ± 0.05	4.47 ± 0.07	44.4 ± 1.2	43.2 ± 1.8	117.1 ± 0.4	1050.4 ± 35.9
6.0	6.27 ± 0.23	5.38 ± 0.23	51.9 ± 0.3	51.9 ± 0.3	139.9 ± 5.5	1241.0 ± 10.8
8.0	7.95 ± 0.26	6.52 ± 0.50	68.4 ± 0.0	68.4 ± .00	173.6 ± 10.1	1647.5 ± 7.2
10.0	10.26 ± 0.24	9.20 ± 0.42	85.1 ± 1.6	85.1 ± 1.6	233.5 ± 7.7	2064.7 ± 25.9
12.0	12.09 ± 0.24	10.47 ± 0.44	101.9 ± 0.6	101.9 ± 0.6	270.8 ± 7.9	2467.6 ± 7.2
14.0	14.2 ± 0.23	12.71 ± 0.59	120.1 ± 1.0	120.1 ± 1.0	323.0 ± 8.4	2892.1 ± 24.9
16.0	15.85 ± 0.25	14.00 ± 0.53	139.1 ± 1.2	139.1 ± 1.2	358.4 ± 7.7	3338.1 ± 28.8
18.0	17.95 ± 0.20	16.06 ± 0.83	158.2 ± 1.0	157.1 ± 1.6	408.1 ± 10.9	3784.2 ± 31.4
20.0	19.86 ± 0.24	18.29 ± 0.43	175.6 ± 0.6	175.6 ± 0.6	457.7 ± 6.2	4215.8 ± 14.4
7.94*	8.12 ± 0.10	6.67 ± 0.05	65.9 ± 0.6	65.9 ± 0.6	177.4 ± 1.6	1582.0 ± 14.0
11.2	11.26 ± 0.07	9.74 ± 0.17	95.5 ± 1.0	92.9 ± 1.2	252.1 ± 2.5	2237.0 ± 26.1

*The last two concentrations were only tested against *C. pomonella* and *E. ceratoniae* eggs.

Table 3. Probit analyses of mortality for eggs of *P. interpunctella, E. elutella, A. transitella, L. serricorne, T. castaneum*, and *C. hemipterus* after exposure to a mixture of propylene oxide and carbon dioxide (8:92) at 25°C and normal atmospheric pressure for 24 h. Lethal doses are concentration x time (CT) (mg h/liter) products.

Species	n	Slope ± SE	LD_{50}	LD_{95} (95% CI)	LD_{99}	χ^2
P. interpunctella	4600	7.7 ± 0.25	47.4 (45.1-49.4)c*	77.7 (73.3-83.5)	95.3 (88.1-105.5)c	19.8
E. elutella	4696	7.18 ± 0.25	49.7 (46.6-52.5)c	84.2 (78.9-91.4)	104.7 (95.9-117.9)bc	21.6
A. transitella	5468	5.6 ± 0.15	51.2 (47.5-54.6)c	100.6 (95.6-115.9)	133.1 (115.5-162.1)b	60.4
L. serricorne	5216	11.6 ± 0.27	71.2 (68.9-73.4)b	98.7 (94.6-104.6)	113.1 (106.5-122.2)b	36.6
T. castaneum	3969	7.8 ± 0.44	174.8 (133.8-195.5)a	284.8 (251.9-389.3)	348.6 (291.7-581.4)a	60.5
C. hemipterus	6514	7.0 ± 0.16	193.4 (180.6-205.3)a	331.7 (304.3-373.1)	414.8 (369.4-488.7)a	49.7

*LD values within a column followed by different letters are significantly different based on overlap of 95% CI.

Table 4. Comparisons of lethal doses (concentration x time) required to kill 50, 95, or 99% of *P. interpunctella* eggs* to those required to kill eggs of *E. elutella, A. transitella, L. serricorne, T. castaneum,* and *C. hemipterus*.

Lethal dose ratios	*E. elutella*	*A. transitella*	*L. serricorne*	*T. castaneum*	*C. hemipterus*
			(95% CI)		
LD_{50}	1.05 (1.02-1.08)	1.08 (1.05-1.11)	1.50 (1.47-1.54)	3.69 (3.55-3.84)	4.09 (3.98-4.19)
LD_{95}	1.84 (1.04-1.13)	1.29 (1.24-1.35)	1.27 (1.23-1.32)	3.67 (3.50-3.85)	4.27 (4.11-4.44)
LD_{99}	1.09 (1.04-1.17)	1.39 (1.31-1.49)	1.00 (0.92-1.08)	3.66 (3.41-3.92)	4.35 (4.13-4.89)

*Because *P. interpunctella* eggs had consistently lower LD values indicating it was the most susceptible, lethal doses of other species were compared to those of *P. interpunctella*.

Table 5. Probit analyses of mortality for eggs of *P. interpunctella*, *A. transitella*, *T. castaneum*, and *C. hemipterus* after exposure to propylene oxide alone at 25°C and normal atmospheric pressure for 24 h. Lethal doses are concentration x time (CT) (mg h/liter) products.

Species	n	Slope ± SE	LD_{50}	LD_{95} (95% CI)	LD_{99}	χ^2
P. interpunctella	6717	10.7 ± 0.25	54.5 (53.3-55.6)c*	77.7 (75.2-80.7)	90.0 (86.1-94.8)b	13.6
A. transitella	6189	3.8 ± 0.16	41.1 (22.5-52.6)b	110.6 (87.2-194.6)	166.7 (118.3-431.8)a	116.6
T. castaneum	2466	9.4 ± 0.57	184.3 (155.1-216.2)a	274.8 (229.7-467.0)	324.5 (258.4-672.0)a	46.5
C. hemipterus	5052	6.6 ± 0.28	212.1 (159.1-245.1)a	376.7 (322.5-525.3)	477.9 (387.2-804.2)a	89.5

*LD values within a column followed by different letters are significantly different as determined by overlap of 95% CI.

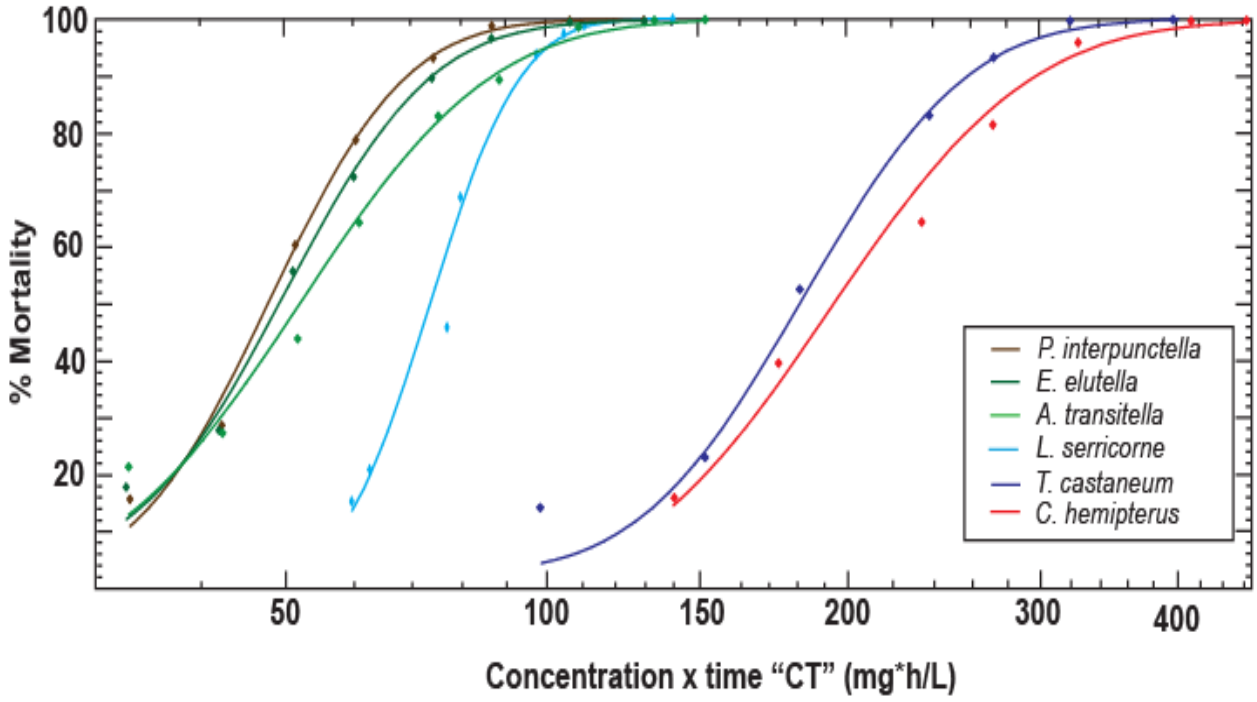
Table 6. Comparisons of LD_{50} and LD_{99} of *P. interpunctella, A. transitella, T. castaneum*, and *C. hemipterus* eggs when exposed to PPO alone or in combination with 92% CO_2 for 24 h.

Species	LD_{50}		LD_{99}	
	PPO alone	PPO + 92% CO_2	PPO alone	PPO + 92% CO_2
*P. interpunctella**	54.5	47.4	90.0	95.3
	(53.3-55.6)a	(45.1-49.4)b	(86.1-94.8)a	(88.1-105.5)a
A. transitella	41.1	51.2	166.7	133.1
	(22.5-52.6)a	(47.5-54.6)a	(118.3-431.8)a	(115.5-162.1)a
T. castaneum	184.3	174.8	324.5	348.6
	(155.1-216.2)a	(133.8-195.5)a	(258.4-672.0)a	(291.7-581.4)a
C. hemipterus	212.1	193.4	477.9	414.8
	(159.1-245.1)a	(180.6-205.3)a	(387.2-804.2)a	(369.4-488.7)a

*LD_{50} or LD_{99} values within a column for each species followed by different letters are significantly different as determined by overlap of 95% CI.

Figure caption

Fig. 1. Dose response relationships of *C. hemipterus*, *T. castaneum*, *L. serricorne*, *A. transitella*, *E. elutella*, and *P. interpunctella* eggs to propylene oxide in a mixture of propylene oxide and CO_2 (8:92). All fumigations were conducted at 25°C and normal atmospheric pressure for 24 h.


100
80
60
40
20
% Mortality
50
100
150
200
300
400
Concentration x time "CT" (mg*h/L)
P. interpunctella
E. elutella
A. transitella
L. serricorne
T. castaneum
C. hemipterus

CHAPTER VII

CONCLUSIONS

Postharvest chamber fumigation is a critical element of the ≈$1.34 billion California walnut industry that produces nearly all the walnuts produced in the United States. As a result of regulatory phaseout of methyl bromide (MeBr), the California walnut industry has transitioned to using sulfuryl fluoride (SF) for postharvest disinfestation. However, SF at the current recommended label rate cannot achieve adequate control of several walnut pests. This work was conducted in the context of overcoming ovicidal deficiencies of SF during postharvest fumigations. The first approach taken investigates a possible mechanistic relationship between the respiratory structures on the surface of the chorion and relative fumigant efficacy. Abundance of respiratory structures on the surfaces of chorions, and structures and thicknesses of the chorions of various stored-product insect eggs were compared. The second approach was to provide efficacy data of propylene oxide (PPO) against key walnut pests for development of a SF-PPO blend to meet disinfestation requirements for walnut pests during reduced and normal atmospheric pressure (NAP) fumigations. Dose responses of key walnut pests, namely, *C. hemipterus*, *T. castaneum*, *L. serricorne*, *P. interpunctella*, *E. elutella*, *A. transitella*, and *C. pomonella* to PPO were established with PPO applied in combination with 100 mmHg or carbon dioxide at NAP.

Comparisons of respiratory structures showed that presence, abundance, distribution, and location of respiratory structures, i.e., aeropyles and micropyles, varied across species. *C. hemipterus* egg had two aeropyles and no micropyles; *T. castaneum* eggs did not have aeropyles or micropyles; *P. interpunctella*, *A. transitella* as well as *L. serricorne* eggs had many aeropyles and several micropyles; and each *E. elutella* egg had many aeropyles and a single micropyle. Respiratory structure normalized areas, i.e., aeropylar areas open to the ambient atmosphere were 990.1, 52.1, 20.7, 5.5, and 2.2 μm^2, in *L. serricorne*, *P. interpunctella*, *E. elutella*, *A. transitella*, and *C. hemipterus*, respectively. Except for *L. serricorne*, where micropylar area was ≈7% of the total surface area open to the ambient atmosphere, micropylar contribution to the total surface area in other eggs studied was negligible (<1%). Chorion composition was unique in each species studied and thicknesses of the chorionic layers varied across species. *A. transitella* had the thickest chorion. *C. hemipterus* and *T. castaneum* had greater intrachorionic air space compared to *P. interpunctella* and *A. transitella* that had greater number of aeropyles. A study that used osmium tetroxide as a model to develop methods for quantifying estimated amounts of gas entering the egg via chorionic and respiratory structures seemed to show greater presence of osmium tetroxide in areas where aeropyles are localized. Although confirmatory measurements of fumigant diffusion into eggs are needed, these findings suggest that species-specific ovicidal efficacies are related, at least in part, to the surface morphology of eggs and that chorionic respiratory structures differentially affect fumigant penetration and/or uptake.

The study on efficacy of PPO against eggs of seven postharvest insect pests, namely, *C. hemipterus*, *T. castaneum*, *P. interpunctella*, *E. elutella*, *L. serricorne*, *C.*

pomonella, and *A. transitella* at 100 mmHg showed that PPO is an effective ovicide. Mortality tests on all insect species resulted in LC_{99} values ranging from 24.2-167.9 mg/liter. Corresponding CT (concentration x time) products were 48.4-674.4 mg h/liter. Generally, PPO was more toxic to lepidopteran eggs than to coleopteran eggs, but *L. serricorne* was the exception. Based on LD_{50} values from the 2- and 4-h fumigations conducted at 100 mmHg, the tolerance to PPO for the species tested in decreasing order was *C. hemipterus* > *T. castaneum* > *P. interpunctella* ≥ *E. elutella* ≥ *L. serricorne* > *C. pomonella* ≥ *A. transitella*. Postembryonic stages of all species did not survive the concentrations of PPO that killed eggs of respective species. The concentrations of PPO required to control eggs of all species of insects tested are much lower than the FDA recommended dose for pasteurization of almonds, i.e. 500 mg/liter for 4 h. Based on the results of this study, a blend of 85 mg/liter of PPO and 8 mg/liter of SF for 2-h fumigation at 100 mmHg is likely effective against all field and storage pests infesting walnuts. This suggests that the blend could be a used as a possible MeBr alternative for fumigation of walnuts in scenarios where rapid disinfestations are required, e.g. during harvest and walnuts are to be disinfested for export or when large amounts of walnuts are being harvested and rapid disinfestations are required to prepare product for storage.

Studies to evaluate the efficacy of PPO alone against *C. hemipterus, T. castaneum*, *P. interpunctella*, and *A. transitella* or when mixed with CO_2 (8: 92) against *C. hemipterus, T. castaneum, P. interpunctella, E. elutella, L. serricorne, C. pomonella, A. transitella,* and *E. ceratoniae* at NAP also showed that PPO is a potent ovicide. In combination with CO_2, mortality tests on all insect species resulted in LC_{99} values ranging from 4.0-17.3 mg/liter. Corresponding CT products were 95.3-414.8 mg h/liter.

Generally, PPO was more toxic to lepidopteran eggs than to coleopteran eggs of the species tested, but *L. serricorne* was the exception. Based on the results of this study, ≈18 mg/liter of PPO mixed with CO_2 (8: 92) will kill eggs of all field and storage insect pests of walnuts.

Based on the results of efficacy data of PPO at reduced pressure (100 mmHg) and NAP, concentrations of PPO required to control eggs of all species tested were much lower than the FDA recommended PPO concentration for almond pasteurization, i.e. 500 mg/liter for 4 h. This suggests that at these lower concentrations of propylene oxide, i.e. 85 mg/liter at 100 mmHg or 18 mg/liter in combination with CO_2 at NAP, the residue concern of PPO and propylene chlorohydrins may be minimal. Absorbance of PPO by in-shellwalnuts fumigated using 85 mg/liter or 18 mg/liter of PPO is recommended to determine compensatory increase after absorbance in order to maintain these concentrations in the headspace during fumigations. Subsequently, evaluation of a blend comprising 8 mg/liter of SF and 85 mg/liter of PPO during 2-h fumigation at 100 mmHg and 8 mg/liter of SF and 18 mg/liter of PPO (in combination with CO_2) during 24-h fumigation at NAP against key walnut pests is recommended. Studies aimed at determining the residues of PPO and propylene chlorohydrins in in-shelland shelled walnuts are needed to determine whether the residues are within acceptable limits. The concentrations of PPO found during residue testing must take into account the absorbance. Results of these two studies represent critical and initial steps in formulating blends of SF-PPO to meet disinfestation requirements of the California walnut industry. The species studied also infest other dried fruits and nuts besides walnuts. Therefore, the PPO efficacy data from these two studies could be used to explore ways to disinfest other

commodities as well. Commodity specific absorbance and residue testing would be required to determine commodity specific SF-PPO blend.

Based on results of egg morphology data, a possible link between the respiratory structures on the surface of the egg chorion and relative fumigant efficacy is suggested. Future studies should be aimed at systematically and quantitatively exploring respiratory structure vs. chorionic diffusion using microscopic and molecular marker techniques, in the context of species-specific fumigant efficacies. In addition, respiratory mechanism of stored product insect eggs and ways to manipulate them with respect to fumigant efficacy needs to be researched.

In conclusion, this research has added novel knowledge on a possible mechanistic relationship between respiratory structures present on the surface of the chorion and relative efficacy of fumigants. Data on efficacy of PPO against key pests of walnuts is a critical initial step in formulating SF-PPO blends that are an alternative to MeBr in postharvest fumigation of walnuts.

APPENDICES

Tables provided in this section summarize the toxciological data in relation to egg respiratory parameters that may influence gas exchange. Toxicological data of PPO presented in the tables are taken from chapters V and VI of this dissertation and that of SF is obtained from the work conducted by Dr. Spencer Walse at the San Joaquin Valley Agricultural Sciences Center USDA-ARS, Parlier, CA. Egg respiratory parameters data is taken from chapters III and IV of this dissertation.

Table 1. Lethal doses (concentration x time) of SF and PPO that are required to kill 50% of egg samples at reduced pressure and parameters of insect eggs that may influence gas exchange.

Species	LD_{50} (mg h/liter)		Respiratory parameters			
	SF (15.6°C)	PPO (25°C)	Number of Aeropyles/egg	Number of Aeropyles/μm^2	Aeropylar Area (μm^2)	Surface Area-to-Volume Ratio
C. hemipterus	3319.8	261.8	2.00 ± 0.0	$2.0 \times 10^{-6} \pm 3.5 \times 10^{-8}$	2.16 ± 0.19	$17.0 \times 10^{-3} \pm 2.2 \times 10^{-4}$
T. castaneum	599.7	79.9	0.0	0.0	0.0	$24.9 \times 10^{-3} \pm 1.6 \times 10^{-4}$
L. serricorne	-	42.3	291,200 ± 13,145	0.87 ± 0.043	990.1 ± 180.4	$24.1 \times 10^{-3} \pm 2.9 \times 10^{-4}$
P. interpunctella	330.7	46.9	13.6 ± 0.25	$5.2 \times 10^{-5} \pm 1.9 \times 10^{-7}$	52.1 ± 4.3	$26.8 \times 10^{-3} \pm 1.7 \times 10^{-4}$
E. elutella	-	42.0	17.24 ± 0.79	$2.9 \times 10^{-5} \pm 1.4 \times 10^{-6}$	20.71 ± 1.39	$13.9 \times 10^{-3} \pm 1.3 \times 10^{-4}$
A. transitella	127.1	22.1	7.24 ± 0.36	$1.06 \times 10^{-5} \pm 5.6 \times 10^{-7}$	5.50 ± 0.34	$14.9 \times 10^{-3} \pm 1.3 \times 10^{-4}$
C. pomonella	268.4	23.6	140	-	-	-

Source: S.W., unpublished data, Cônsoli et al. 1999.

Table 2. Lethal doses (concentration x time) of SF and PPO that are required to kill 50% of egg samples at normal atmospheric pressure and parameters of insect eggs that may influence gas exchange.

Species	LD_{50} (mg h/liter)		Respiratory parameters			
	SF (15.6°C)	PPO (25°C)	Number of Aeropyles/egg	Number of Aeropyles/μm^2	Aeropylar Area (μm^2)	Surface Area-to-Volume Ratio
C. hemipterus	5320.9	193.4	2.00 ± 0.0	$2.0 \times 10^{-6} \pm 3.5 \times 10^{-8}$	2.16 ± 0.19	$17.0 \times 10^{-3} \pm 2.2 \times 10^{-4}$
T. castaneum	770.3	174.8	0.0	0.0	0.0	$24.9 \times 10^{-3} \pm 1.6 \times 10^{-4}$
L. serricorne	371.8*	71.2	291,200 ± 13,145	0.87 ± 0.043	990.1 ± 180.4	$24.1 \times 10^{-3} \pm 2.9 \times 10^{-4}$
P. interpunctella	452.2	47.4	13.6 ± 0.25	$5.2 \times 10^{-5} \pm 1.9 \times 10^{-7}$	52.1 ± 4.3	$26.8 \times 10^{-3} \pm 1.7 \times 10^{-4}$
E. elutella	164.1**	49.7	17.24 ± 0.79	$2.9 \times 10^{-5} \pm 1.4 \times 10^{-6}$	20.71 ± 1.39	$13.9 \times 10^{-3} \pm 1.3 \times 10^{-4}$
A. transitella	270.0	51.2	7.24 ± 0.36	$1.06 \times 10^{-5} \pm 5.6 \times 10^{-7}$	5.50 ± 0.34	$14.9 \times 10^{-3} \pm 1.3 \times 10^{-4}$
C. pomonella	238.0	-	140	-	-	-

Source: S.W., unpublished data, Cônsoli et al. 1999, Su and Scheffrahn 1990, Baltaci et al. 2009

*Fumigation tests were conducted at 26.5°C

**Fumigation tests were conducted at 25°C

VITA

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